action of April 4, 2000, has now been carefully studied. Reconsideration and allowance are hereby respectfully urged.

Briefly, the present invention relates to cDNA sequences which encode polypeptides that bind to TRAF2 and modulate activity of NF-κB as well as the polypeptides encoded by those DNA sequences. Preferably, the polypeptide is NIK. The invention also relates to antibodies, methods of identification and pharmaceutical compositions, as well as antisense DNA.

It is noted with appreciation that the examiner has reconsidered and withdrawn the election/restriction requirement in light of applicant's amendments to the claims and applicant's arguments. Furthermore, the examiner has accepted applicant's sequence listing.

The examiner has brought to applicant's attention U.S. patents 5,843,721 and 5,844,073 which are not available as prior art. However, these patents claim the same invention as being claimed in the present application. Accordingly, applicant intends to file a request for interference with these patents in accordance with 37 C.F.R. §1.607 within the very near future.

Claims 22, 26-29, 31-39, 53, 55 and 58-60 have been rejected under 35 USC 112, second paragraph, as being indefinite. The examiner states that claim 22 recites "[a]ntibodies ... or derivative thereof according to claim 19 or 20" and that as the claim reads on multiple antibodies, fragments and derivatives,

there is ambiguity as to whether the claim reads on one particular antibody or several different antibodies.

First of all, it is apparent that the PTO erred in entering applicant's supplemental preliminary amendment of October 2, 1998, insofar as claim 22 is concerned. It was intended that everything in the claim from "or 18" to the end of the claim be deleted. Claim 22 has now been rewritten in amended form which includes this previously requested amendment, thus obviating the first part of this rejection. The new form of claim 22 now specifies "An antibody, fragment or derivative thereof", thus eliminating any ambiguity which the examiner may have noted. Claim 50 has been similarly amended. Reconsideration and withdrawal of this part of the rejection is respectfully urged.

Claims 26-28 are considered vague and indefinite in the term "TRAF2 modulated/mediated effect on cells". This part of the rejection is respectfully traversed.

Claim 26 has now been amended to refer to "A method for modulating the effect of TRAF2 on cells ...". Thus, the term "modulated/mediated effect" to which the examiner has objected no longer appears in the claim. Claims 27 and 28 have now been deleted, thus obviating this part of the rejection. Reconsideration and withdrawal of this part of the rejection are therefore respectfully urged.

Claims 27 and 29 have been rejected for lack of antecedent basis. These claims have now been deleted, thus obviating this part of the rejection.

The examiner has objected to claim 31 for lack of antecedent basis for the language "said protein is NIK or at least one of the NIK isoforms, analogs, fragments and derivatives thereof."

Claim 31 has now been deleted in favor of new claim 61 rewritten in a form which eliminates all ambiguity and provides sufficient antecedent basis for every term therein. Accordingly, this part of the rejection has now been obviated.

The examiner states that in claim 53 there is lack of antecedent basis for the reference to NIK (SEQ ID NO:7).

Claim 51, from which claim 53 depends, has now been amended to refer to SEQ ID NO:6, rather than SEQ ID NO:3, which was only a portion of NIK. SEQ ID NO:6 is the nucleotide sequence which encodes NIK, whose amino acid sequence is SEQ ID NO:7. Claim 53 has now been amended to specifically refer to the polypeptide encoded by the nucleotide sequence of SEQ ID NO:6 and defining that as "NIK (SEQ ID NO:7)". Accordingly, this part of the rejection has now been obviated.

The examiner states that claim 55 is vague and indefinite in the use of the phrase "moderately stringent conditions" since the metes and bounds of the term "moderately"

in this context is uncertain. This part of the rejection is respectfully traversed.

MPEP §2173.02 states:

> The examiner's focus during examination of claims for compliance with the requirement for definiteness of 35 U.S.C. §112, second paragraph is whether the claim meets the threshold requirements of clarity and precision, not whether more suitable language or modes of expression are available. ...
>
> The essential inquiry pertaining to this requirement is whether the claims set out and circumscribe a particular subject matter with a reasonable degree of clarity and particularity. Definiteness of claim language must be analyzed, not in a vacuum, but in light of:
>
> (A) the content of the particular application disclosure;
>
> (B) the teachings of the prior art; and
>
> (C) the claim interpretation that would be given by one possessing the ordinary level of skill in the pertinent art at the time the invention was made.

At the time the present invention was made, the metes and bounds of moderate stringency were known to those of skill in the art, even though there may be some variation in the means for providing roughly the same level of stringency either at the hybridization stage or at the wash stage. U.S. patent 5,026,636, relevant pages of which are attached, was available at the time the invention was made and defines moderate stringency as conditions that allow detection of

nucleotide sequences at least approximately 75% homologous to the probe (column 4, lines 50-65). This patent further teaches that moderately stringent conditions for a particular probe, when seeking a specified degree of homology, may be readily determined by those of skill in the art using, for example, the reference text, Nucleic Acid Hybridisation: A Practical Approach, Hames and Higgins, eds., IRL Press, Washington (1985) or the scientific publication, Wood et al, Proc. Natl. Acad. Sci. USA 82:1585-1588 (1985) (a copy of which is attached hereto), for guidance. Chapter 4 of Nucleic Acid Hybridisation: A Practical Approach, on quantitative filter hybridization, a copy of which is attached hereto, teaches the various factors affecting hybrid stability through a calculation of melting temperature (T_m) of the hybrid using the standard equation (Equation 7 on page 80) and factoring in the percent mismatch (percent identity) that is sought. The calculation of T_m takes into account the molarity of the monovalent cation (i.e., sodium in SSC solution). Accordingly, those of skill in the art would recognize and understand what the metes and bounds of the conditions needed for moderate stringency hybridization to detect a hybrid with a specified percent identity, e.g., 75%.

Indeed, U.S. patents 4,968,607 (50°C, 2 X SSC; column 10, lines 39-40), 5,171,675 (50°C, 2 X SSC; column 6,

line 49), 5,198,342 (50°C, 2 X SSC; column 9, lines 54-55), 5,262,522 (50°C, 2 X SSC; column 15, lines 8-9), and 5,237,051 (60°C, 1 X SSC; column 5, lines 24-27), which are available as prior art, demonstrate that those in the art were able to determine and define moderately stringent conditions based on the knowledge and skill at that time. All have claims which include the term "moderate stringency". Relevant pages of the above-cited U.S. Patents are attached hereto. According to the Federal Circuit Court of Appeals, it is relevant to the issue of definiteness that the criticized words are used frequently in patent claims. See *Andrew Corp. v. Gabriel Electronics*, 6 USPQ2d 2010, 2012-13 (Fed. Cir. 1988). And see also *Ex parte Brian*, 118 USPQ 242, 245 (POBA 1958), where it states:

> Since the claims under consideration are similar to those in the patents, we do not feel disposed to reject them and thus upset such a long established practice in the particular art under consideration.

In other words, the very fact that "moderate stringency" claims have been repeatedly allowed in the past is reason to consider them definite. This is true, not only because the use of such claims by many different inventors and examiners is evidence that the terminology is considered sufficiently definite by the art, but also because a reinterpretation of the definiteness of such claims by the PTO casts a shadow of

doubt on previously issued "moderate stringency" claims, even though such claims are entitled to a presumption of validity.

Furthermore, the widely used reference text, Current Protocols in Molecular Biology, eds. Ausubel et al, John Wiley & Sons, Inc., (1987-1998) on page 2.10.11 (Supplement 26, 1994[1]), a copy of which is also attached, guides those of skill in the art how to use a rational approach at determining "moderate stringency" wash conditions by calculating the decrease in temperature required using the correlation for decrease in T_m per percent mismatch.

Accordingly, in view of the teachings of the prior art, it is urged that the claim interpretation to be given to the term "hybridization under moderately stringent conditions" are those conditions which would permit detection of nucleotide sequences at least approximately 75% homologous. Thus, the scope of the invention sought to be patented can be determined from the language of the claims with a reasonable degree of certainty. Reconsideration and withdrawal of this rejection are, therefore, respectfully urged.

The examiner has objected to claim 58 as it depends from claim 55 which recites a variety of DNA sequences of different compositions and lengths. The examiner states that it

[1] A copy of page 2.6.1 is also attached which shows that Supplement 26 is dated 1994).

is unclear how the variety of sequences recited in claim 55 can all encode the same proteins.

Claim 55 has now been amended to change the reference to SEQ ID NO:3 to read SEQ ID NO:6. Furthermore, claim 58 has now been amended to more exactly correspond to the language of paragraph 55(vi). It is believed that these amendments should now obviate this part of the rejection.

The examiner states that claim 59 is indefinite in referring to a DNA sequence which encodes a DNA sequence.

Claim 59 has now been amended to correct this inadvertent error.

The examiner states that claim 60 recites "said antisense oligonucleotide being capable of effectively blocking the translation of said mRNA." The examiner states that the term "capable" as used in this phrase is vague and indefinite since it suggests latent function of the antisense oligonucleotide which may or may not be functional under certain conditions. This part of the rejection is respectfully traversed.

The term "capable" as used in claim 60 does not refer to a latent function. It refers to a property of the oligonucleotide. Obviously, an oligonucleotide may be covered by claim 60 regardless of whether or not it is being used to block the translation of mRNA. However, it is a property of the oligonucleotide that it is capable of effectively blocking the

translation of mRNA. The term is neither vague nor indefinite as any potentially infringing oligonucleotide either has this capability or does not. This is no more indefinite than stating that water is "capable of" boiling at 100°C. If the examiner prefers, applicant would be willing to change the end of claim 60 to read "said antisense oligonucleotide having the ability to effectively block the translation of said mRNA." Reconsideration and withdrawal of this part of the rejection are therefore respectfully urged.

The examiner states that claims 32-39 provide for the use of a pharmaceutical composition, but as the claims do not set forth any steps involved in the method or process, it is unclear what method or process applicant is intending to encompass. The examiner states that a claim is indefinite when it merely recites the use without any active positive steps to limit how this use is actually practiced. The examiner further rejects claims 32-39 under 35 USC 101 because a claimed recitation of a use without setting forth any steps involved in the process result in an improper definition of a process. This rejection is respectfully traversed.

Claims 32-39, of which only claims 32 and 33 remain in the case, are not "use" claims. They are clearly pharmaceutical composition claims. The statement that the composition is "for the modulation of the effect of TRAF2 on cells", is merely a

statement of intended use as appears in all composition claims. Composition claims fully comply with 35 USC 101. As claims 32 and 33 are not claiming a use but are claiming a composition, these rejections are not applicable. Reconsideration and withdrawal thereof are respectfully urged.

Claims 13-16 and 20-60 have been rejected under 35 USC 112, first paragraph, as failing to comply with the written description requirement. The examiner states that claim 51 and those claims dependent therefrom read on a polypeptide that binds to TRAF2 and modulates the activity of NF-κB comprising the amino acid sequence of a fragment, analog or derivative of the amino acid sequence of SEQ ID NO:2, an amino acid sequence encoded by the nucleotide sequence of SEQ ID NO:3 (now changed to SEQ ID NO:6), or the amino acid sequence of SEQ ID NO:5, as well as analogs and derivatives. The examiner states that the specification and claims do not indicate what distinguishing attributes are shared by the members of the genus. The examiner states that the specification does not provide any guidance as to what changes should be made in order to isolate the recited fragments of the claimed polypeptides, analogs of said polypeptides and fragments, and derivatives of said polypeptides which retain the ability to bind TRAF2 and modulate the ability of NF-κB. The examiner states that structural features that could distinguish compounds in the genus from others in the

polypeptide class are missing from the disclosure and no common structural attributes identify the members of the genus. The examiner states that since the disclosure fails to describe the common attributes or characteristics that identified numbers of the genus, and because the genus is highly variant, the recited amino acid sequences of SEQ ID NO:2 and 5 and the amino acid encoded by the nucleotide sequence of SEQ ID NO:3 are not sufficient to describe the genus and that one skilled in the art would reasonably conclude that the disclosure fails to provide a representative number of species to describe the genus, and, thus, applicant was not in possession of the claimed genus. This rejection is respectfully traversed.

First of all, it is noted that the examiner has not required restriction among the species of the DNA sequences of SEQ ID NO:1, SEQ ID NO:6, and SEQ ID NO:4. This is undoubtedly because MPEP §803.04 states that it is the policy of the Office to permit a reasonable number of such nucleotide sequences to be claimed in a single application. These three sequences are being claimed in a Markush type generic claim because they all share a common utility and they all encode proteins which bind to TRAF2 and modulate activity of NF-κB. MPEP §803.02 states:

> If the members of the Markush group are sufficiently few in number or so closely related that a search and examination of the entire claim can be made without serious burden, the examiner must examine all claims on the merit, even though they are directed

> to independent and distinct inventions. In such a case, the examiner will not follow the procedure described below and will not require restriction.

If Markush claim 55 was divided into three independent claims, one drawn to SEQ ID NO:1 fragments and analogs, the second directed to the SEQ ID NO:6 fragments and analogs, and the other directed to SEQ ID NO:4 fragments and analogs, then the examiner would still examine all of these claims in light of MPEP §803.04. What then is the purpose of objecting to the Markush type claim 55? It is as easy for the examiner to examine these three species in a Markush claim as in independent claims. Accordingly, it is requested that this objection to the generic claim be withdrawn.

Certainly, for each of these three SEQ ID numbers, the DNA itself and the DNA encoding fragments, analogs and derivatives, share structural attributes and are appropriately considered to be within a single genus. Furthermore, if one has described each of the species which, in aggregate, comprise the entire genus, then one has described the genus. The members of the genus are not highly variant as they all share the common attribute of encoding a polypeptide that binds to TRAF2 and modulates activity of NF-κB. This is true of the three sequences of claims 55(i), (ii) and (iii). It is also true of the fragment of paragraph 55(iv). A fragment is not "highly variant" as every nucleotide of the fragment must appear in the full sequence.

Similarly, DNA sequences capable of hybridization under moderately stringent conditions must still encode a polypeptide that binds to TRAF2 and modulates the activity of NF-κB, and, as discussed above, this only includes analogs of about 75% identity. This is not so highly variant as to preclude written description.

Rejections under the written description requirement of 35 U.S.C. §112 should be analyzed in accordance with the "Revised Interim Guidelines for Examination of Patent Applications under the 35 U.S.C. §112, ¶1, 'Written Description' Requirements; Request for Comments", published at FR71427 on December 21, 1999, as well as the "Revised Interim Written Description Guidelines Training Materials", which have been published at the PTO website. Analogous to Examples 9, 10 and 14 of the "Revised Interim Written Description Guidelines Training Materials" relating to hybridization language and product by function language, it is believed that the analysis should proceed as follows.

A review of the full content of the specification indicates that the essential feature of the claimed invention is the isolated nucleic acid for the three specified DNA sequences, fractions thereof which maintain a specific function, and isolated nucleic acids that hybridize to any one of those three sequence ID numbers under moderately stringent

conditions and encode a polypeptide that binds to TRAF2 and modulates the activity of NF-κB. As discussed hereinabove in the context of the indefiniteness rejection, the prior art indicates that hybridization techniques using a known DNA as a probe under moderately stringent conditions were conventional in the art at the time of filing.

Insofar as section (v) of claim 55 is concerned, the claim is drawn to a genus of nucleic acids, all of which must hybridize with one of the specified sequence ID numbers and must encode a protein with the specified activity. The examiner's search of the prior art indicates that those of the presently specified and claimed sequences are novel and unobvious.

For each sequence ID number specified in claim 55, there is a single species disclosed that is within the scope of the claimed genus. There is actual reduction to practice of each of these disclosed species.

Now turning to the genus analysis, it must be determined whether a person of skill in the art would expect substantial variation among species encompassed within the scope of the claims. As discussed above in the indefiniteness rejection, hybridization at moderate stringency is designed to find nucleotides with 75% or more identity with the specified probes. This amount of identity is not "substantial

variation". Example 14 of the Training Materials indicates that variants that are at least 95% identical to a specified sequence ID number and have the required function comply with the written description requirement as there is not substantial variation, and a single species is sufficient to establish that applicant was in possession of the entire genus. It is urged that 75% identity also would not be considered to be "substantial variation". Section II.A.3.a.(2) of the Interim Guidelines states:

> (2) For each claim drawn to a genus:
>
> The written description requirement for a claimed genus may be satisfied through sufficient description of a representative number of species by actual reduction to practice (see (1)(a), above), reduction to drawings (see (2)(b), above), or by disclosure of relevant identifying characteristics, *i.e.*, structure or other physical and/or chemical properties, by functional characteristics coupled with a known or disclosed correlation between function and structure, or by a combination of such identifying characteristics, sufficient to show the applicant was in possession of the claimed genus (see (1)(c), above).

Here, relevant identifying characteristics of the whole genus are disclosed, as similarity to very specific structures is required. Functional characteristics are specified in that a polypeptide encoded by the DNA sequence must bind to TRAF2. There is a known or disclosed correlation between the function and structure, i.e., structure having at least 75% identity

(or encoded by a DNA which hybridizes under conditions of moderate stringency) would be expected to have a sufficiently homologous structure to have the same function and an assay is disclosed in order to test this.

Thus, by analogy to Example 9 of the Training Materials, a representative number of species is disclosed, since moderately stringent hybridization conditions in combination with the coding function of DNA and the level of skill and knowledge in the art are adequate to determine that applicants were in possession of the claimed invention. This is not a situation as in claim 2 of Example 10 of the Training Materials, which does not describe any stringency conditions at all, as those conditions would be expected to yield structurally unrelated nucleic acid molecules. Moderate stringency, which is expected to yield only molecules with 75% or more identity, must by definition, yield structurally-related nucleic acid molecules. If a claim specifying 75% identity would be considered to meet the written description requirement, then a claim specifying hybridization at moderate stringency should also be considered to meet the written description requirement.

The same analysis is applicable to claim 51. Accordingly, reconsideration and withdrawal of this rejection are respectfully urged.

Claim 55 and those claims dependent therefrom are included in this rejection as one of ordinary skill in the art would reasonably conclude that hybridization under moderately stringent conditions may result in hybridizations to nucleic acid sequences beyond the description of the nucleic acid sequences provided in the instant specification, and thus applicant was not in possession of the claimed genus. This part of the rejection is respectfully traversed.

The reasons why hybridization under moderately stringent conditions satisfies the first paragraph of 35 USC 112 have already been discussed hereinabove with respect to the previous part of the rejection. Accordingly, reconsideration and withdrawal of this part of the rejection for the reasons discussed therein are hereby respectfully urged.

Claims 27-29, 34 and 40-42 have been rejected under 35 USC 112, first paragraph, as failing to comply with an enablement requirement.

While it is not understood why claims 40-42 were included in this rejection, nevertheless in order to obviate this rejection, claims 27-29, 34 and 40-42 have now been deleted without prejudice toward the continuation of prosecution thereof in a continuing application. The deletion of these claims is not a concession that the rejection is tenable. The deletion is made

without dedication, disclaimer, abandonment, waiver, forfeiture or estoppel.

As to claims 30-39 and 40-42, the examiner states that these claims read on pharmaceutical compositions and methods for the prevention or treatment of a pathological condition associated with NF-κB induction or with any other activity mediated by TRAF2 or by other molecules to which a polypeptide according to claim 51 binds, the method comprising administering an effective amount of a polypeptide according to claim 51. The examiner states that the methods are not supported by the specification in an enabling manner. This rejection is respectfully traversed.

It is believed that the examiner may have erred in referring to claims 30-34 in this rejection. It is believed that the rejection only appears applicable to claims 35-39 and 40-42, as claim 30 is drawn to a method for isolating and identifying a polypeptide, claim 31 is dependent from claim 23 which was not included in the rejection, and the compositions of claims 32 and 34 are not for the prevention or treatment of a pathological condition. Accordingly, none of the examiner's reasoning applies to claims 30-34. Reconsideration and withdrawal of this rejection insofar as claims 30-34 are concerned, are therefore respectfully urged.

Claims 35-39 and 40-42 have now been deleted without prejudice toward the continuation of prosecution thereof in a continuing application. Again, deletion of these claims should not be taken as a concession that the rejection is tenable, and the deletion is expressly made without dedication, disclaimer, waiver, forfeiture, abandonment or estoppel.

It is submitted that all of the claims now present in the case clearly define over the references of record and fully comply with 35 USC 112. Reconsideration and allowance are therefore earnestly solicited.

Respectfully submitted,

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Table 2.10.3 High-Salt Solutions Used in Hybridization Analysis

Stock solution	Composition
20× SSC	3.0 M NaCl/0.3 M trisodium citrate
20× SSPE[a]	3.6 M NaCl/0.2 M NaH_2PO_4/0.02 M EDTA, pH 7.7
Phosphate solution[b]	1 M $NaHPO_4$, pH 7.2[c]

[a]SSC may be replaced with the same concentration of SSPE in all protocols.

[b]Prehybridize and hybridize with 0.5 M $NaHPO_4$ (pH 7.2)/1 mM EDTA/7% SDS [or 50% formamide/0.25 M $NaHPO_4$ (pH 7.2)/0.25 M NaCl/1 mM EDTA/7% SDS]; perform moderate-stringency wash in 40 mM $NaHPO_4$ (pH 7.2)/1 mM EDTA/5% SDS; perform high-stringency wash in 40 mM $NaHPO_4$ (pH 7.2)/1 mM EDTA/1% SDS.

[c]Dissolve 134 g $Na_2HPO_4 \cdot 7H_2O$ in 1 liter water, then add 4 ml 85% H_3PO_4. The resulting solution is 1 M Na^+, pH 7.2.

Designing washes for heterologous hybridization. Calculations of T_m become more critical if heterologous probing is being attempted. If the aim is to identify sequences that are merely related, not identical, to the probe (e.g., members of a multigene family, or a similar gene in a second organism), then it is useful to have an idea of the degree of mismatching that will be tolerated by a "moderate-" or "low-" stringency wash. The best way to approach this is to first establish the lowest temperature at which only homologous hybridization occurs with a particular SSC concentration. Then assume that 1% mismatching results in a 1°C decrease in the T_m (Bonner et al., 1973) and reduce the temperature of the final wash accordingly (for example, if sequences with ≥90% similarity with the probe are being sought, decrease the final wash temperature by 10°C). If the desired degree of mismatching results in a wash temperature of <45°C, then it is best to increase the SSC concentration so that a higher temperature can be used. Doubling the SSC concentration results in a ~17°C increase in T_m, so washes at 45°C in 0.1× SSC and 62°C in 0.2× SSC are roughly equivalent. Note that in these extreme cases it may also be necessary to reduce the hybridization temperature to as low as 45°C (aqueous solution) or 32°C (formamide solution).

This approach sometimes works extremely well (as shown when the heterologous targets are eventually sequenced), but the assumption that a 1% degree of mismatching reduces the T_m of a heteroduplex by 1°C is very approximate. Base composition and mismatch distribution influence the actual change in T_m, which can be anything between 0.5° and 1.5°C per 1% mismatch (Hyman et al., 1973). Unfortunately trial and error is the only alternative to the "rational" approach described here.

Other parameters relevant to hybridization analysis

Length of prehybridization and hybridization incubations. The protocols recommend prehybridization for 3 hr with nitrocellulose and 15 min for nylon membranes. Inadequate prehybridization can lead to high backgrounds, so these times should not be reduced. They can, however, be extended without problem.

Hybridizations are usually carried out overnight. This is a rather sloppy aspect of the procedure, because time can have an important influence on the result, especially if, as described above, an excess amount of a single-stranded probe is being used. The difficulties in assigning values to the parameters needed to calculate optimum hybridization time has led to the standard "overnight" incubation, which in fact is suitable for most purposes. The exception is when hybridization is being taken to its limits, for instance in detection of single-copy sequences in human DNA, when longer hybridization times (up to 24 hr) may improve sensitivity if a single-stranded probe is being used. Note that this does not apply to double-stranded probes, as gradual reannealing results in only minimal amounts of a double-stranded probe being free to hybridize after ~8 hr of incubation.

Formamide hybridization buffers. Formamide destabilizes nucleic acid duplexes, reducing the T_m by an average of 0.6°C per 1% formamide for a DNA-DNA hybrid (Meinkoth and Wahl, 1984) and rather less for a DNA-RNA hybrid (Casey and Davidson, 1977; Kafatos et al., 1979). It can be used at 50% concentration in the hybridization solution, reducing the T_m so that the incubation can be carried out at a lower temperature than needed with an aqueous solution. Originally formamide was used with nitrocellulose membranes as a means of prolonging their lifetime, as the

Nucleic acid hybridisation

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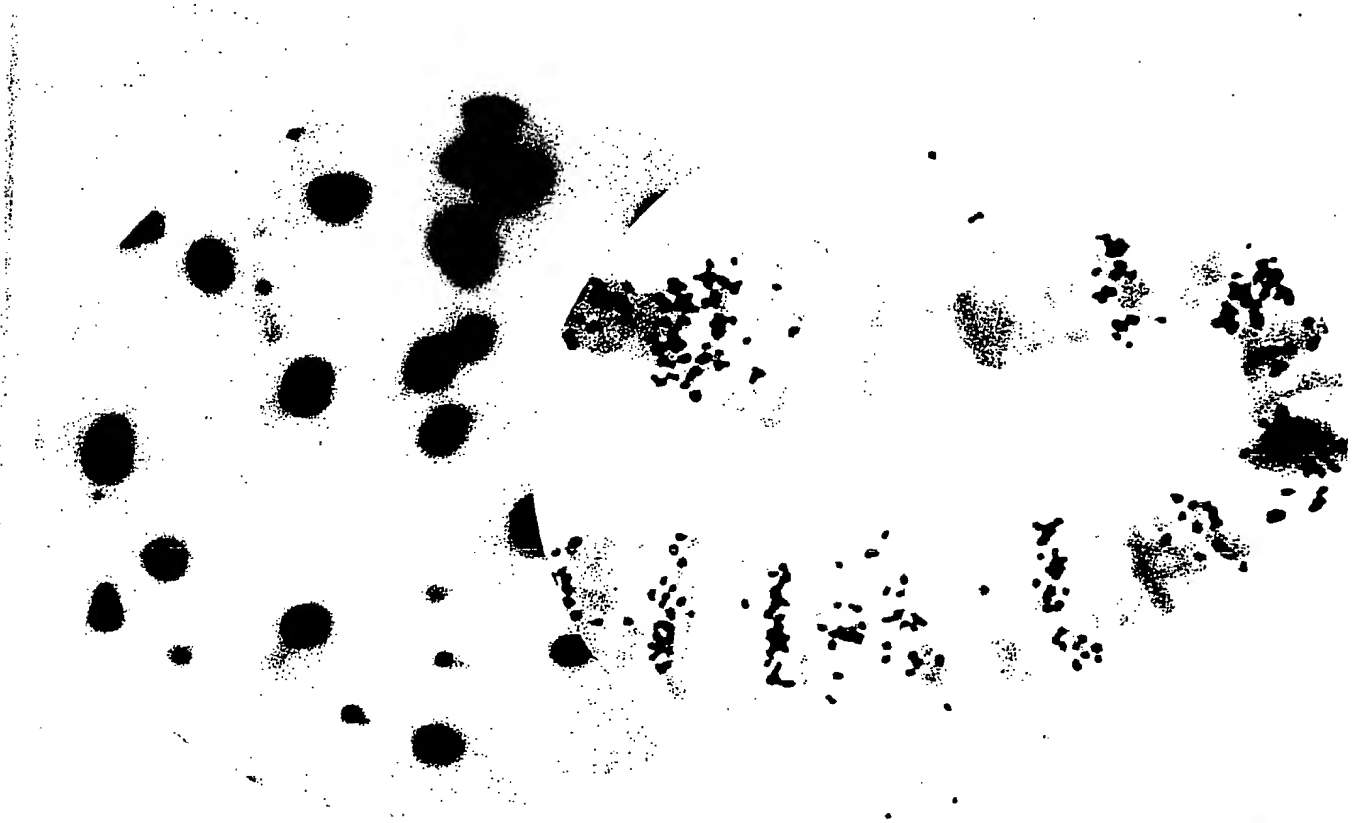

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CHAPTER 4

Quantitative Filter Hybridisation

MARGARET L.M. ANDERSON and BRYAN D. YOUNG

1. INTRODUCTION

An application of nucleic acid hybridisation, which is of central importance to genetic engineering and is finding increasing use in molecular biology, is filter hybridisation. The technique is derived from the classical experiments of Gillespie and Speigelman (1). Denatured DNA or RNA is immobilised on an inert support, for example nitrocellulose, in such a way that self-annealing is prevented, yet bound sequences are available for hybridisation with an added nucleic acid probe. To facilitate analysis, the probe is labelled, often with ^{32}P. Hybridisation is followed by extensive washing of the filter to remove unreacted probe. Detection of hybrids is usually by autoradiography although, when the hybrids are sufficiently radioactive, scintillation counting can be used. The procedure is widely applicable, being used for phage plaque and bacterial colony hybridisation, Southern and Northern blot hybridisation, dot blot hybridisation and hybrid selection (see other chapters in this volume).

While solution hybridisation is the standard method for quantitative measurements of sequence complexity and composition (2), there are practical difficulties when the number of samples is large. By contrast, dot blot hybridisation is ideally suited to the analysis of multiple samples. The technique has the added advantage that it is easy to prepare replicate filters allowing many filter-bound sequences to be analysed at the same time, for example with different probes or under different hybridisation and washing conditions. Dot blot hybridisation can be used qualitatively since it is capable of great discrimination, as exemplified by the ability to distinguish between closely similar members of multigene families (3,4). It can also be used quantitatively with appropriate calibration (5), but it is most commonly used as a semi-quantitative method for determining the relative levels of sequences in different samples. As we shall see, its use is limited by the low rate of hybridisation and by a level of sensitivity which makes it less useful than solution hybridisation for analysing rare sequences.

In this chapter we will first discuss theoretical aspects of filter hybridisation (Sections 2 – 5) and then describe practical aspects (Sections 6 – 14). To make clear the distinction between sequences which are in solution and those which are filter-bound, we will use a different nomenclature from that in the previous chapter. Subscripts 's' and 'f' will be used for nucleic acid in solution and filter-bound nucleic acid, respectively.

2. KINETICS OF FILTER HYBRIDISATION

Nucleic acid hybridisation depends on the random collision of two complementary sequences. As described in the previous chapter, the time course of the reaction in

solution is determined by the concentration of the reacting species and by the second order rate constant, k. The stability of the duplex formed is dependent on its melting temperature, T_m. For hybridisation of perfectly-matched complementary sequences in solution, equations have been derived which describe the process fairly precisely (e.g., Chapter 3, Equations 4, 5 and 10). Detailed investigations have determined the effects of changes in reaction conditions for solution hybridisation, so that values for k and T_m can be calculated with some confidence. In contrast, filter hybridisation has been less extensively studied, and the parameters affecting the rate and extent of reaction are less well understood. Calculations made from solution hybridisation are not necessarily valid for filter hybridisation, although changes in reaction conditions probably have a similar qualitative effect.

The hybridisation of a denatured nucleic acid probe in solution to a filter-bound nucleic acid is a function of two competing reactions, *viz.* the reassociation of sequences in solution and the hybridisation to filter-bound DNA or RNA. (Since the filter-bound nucleic acid is immobilised, reassociation of bound sequences does not occur.) The rate of disappearance of single strands may be expressed by the equation:

$$\frac{-d[C_s]}{dt} = k_1[C_f][C_s] + k_2[C_s]^2 \qquad \text{Equation 1}$$

where C_f is the concentration of filter-bound nucleic acid sequence, C_s is the concentration of nucleic acid probe in solution, k_1 is the rate constant for the hybridisation reaction on the filter and k_2 is the rate constant for the reassociation in solution. The term $k_1[C_f][C_s]$ represents the filter hybridisation while the term $k_2[C_s]^2$ represents reassociation in solution.

A variety of factors affect the rate constants (see Section 3). The rate constant should be the same for both reactions (i.e., $k_1 = k_2$) provided that:

(i) the nucleation rates at the filter and in solution are the same

(ii) the effective molecular weight of the nucleic acid species in solution is smaller than that of the filter-bound nucleic acid, since the rate constant for DNA-DNA reassociation is dependent on the size of the smaller fragment (6,7), at least in solution.

Equation 1 predicts that the initial rate of hybridisation is proportional to the concentrations of both the probe in solution and the filter-bound sequences. When $[C_f]$ is much higher than $[C_s]$, as in dot blots of plasmid DNA (this Chapter, Section 6.2.1 and Chapter 5, Section 3.1) or in hybrid selection (Chapter 5, Section 3.2), the solution reassociation term can be ignored and Equation 1 simplifies to the pseudo-first order reaction:

$$\frac{-d[C_s]}{dt} = k_1[C_f][C_s] \qquad \text{Equation 2}$$

where $[C_f]$ is constant.

On integration, this gives:

$$\frac{[C_s]_t}{[C_s]_0} = e^{-k_1[C_f]t} \qquad \text{Equation 3}$$

where $[C_s]_t$ is the value of $[C_s]$ at time t.

While it has been shown experimentally that the initial hybridisation rate is proportional to $[C_s]$, the relationships in Equations 1 and 2 do not describe exactly the dependency of the hybridisation rate on $[C_f]$. At low values of $[C_f]$, the initial rate of hybridisation is proportional to $[C_f]$, but the rate does not increase linearly at higher values (8 — 10). This is explained by the fact that filter hybridisation depends on two processes, diffusion of the probe to the filter and hybridisation at the filter. It is thought that at low values of $[C_f]$, the hybridisation reaction itself is the rate-limiting step, whereas at high values of $[C_f]$ hybridisation is so fast that the solution surrounding the filter becomes depleted of probe and the overall reaction is then limited by diffusion of the probe to the filter. Flavell *et al.* (9) have shown that at higher values of $[C_f]$ the rate equation should incorporate a term, J, to take diffusion of the probe into account. Therefore, at high values of $[C_f]$, Equation 1 can be replaced by an equation of the form:

$$\frac{-d[C_s]}{dt} = J + k_2[C_s]^2 \qquad \text{Equation 4}$$

where $k_1\,[C_f]\,[C_s] > J > 0$. The diffusion term J is a function of the diffusion coefficient of the probe and the concentration gradient of the probe. The relationship between J and $k^2[C_s]^2$ determines whether reassociation of the probe is an important factor. When J is $\leq k_2[C_s]^2$, reassociation will be significant.

Since many filter hybridisation experiments aim to hybridise the maximum amount of probe to excess sequences on the filter, the relationship given in Equation 4 is important. The overall hybridisation reaction will be speeded up by factors which increase diffusion of the probe to the filter, for example, using a small probe, high incubation temperature, low reaction volume and shaking the reaction vessel. The rate constant k_1 can be determined by two methods.

(i) From initial reaction rates. Rearranging the terms in Equation 2,

$$k_1 = \frac{v_i}{[C_f][C_s]}$$

where v_i is the initial rate of reaction. So in a filter-bound DNA excess reaction, a plot of the reciprocal of the percentage of added probe which has hybridised to the filter *versus* the reciprocal of the time of reaction will give a straight line with a slope of $1/k_1$ (Chapter 3, Section 3.3.1). This holds true for both double-stranded and single-stranded probes, although a small correction may have to be made in the value of $[C_s]$ for reassociation of the probe in solution.

(ii) From measuring $t_{1/2}$, that is, the time when $[C_s]_t/[C_s]_0$ is 0.5. For a filter-bound DNA-excess reaction (pseudo-first order kinetics) a plot of log [fraction of the

probe remaining single-stranded] against the time of reaction will give a straight line. The $t_{½}$ can be read off the graph and substituted in Equation 3 to give:

$$[C_f]t_{½} = \frac{\ln 2}{k_1} \qquad \text{Equation 5}$$

or

$$k_1 = \frac{0.693}{[C_f]t_{½}} \qquad \text{Equation 6}$$

Experimentally, values for k_1 obtained from $t_{½}$ measurements and from initial rate data for hybridisation of a simple DNA probe to filter-bound DNA are in good agreement. However, the values obtained are 10 times lower than those obtained for solution hybridisation of the same DNAs (9). The reason may be that only a fraction of the DNA bound to the filter is accessible for nucleation, although all the DNA can effectively participate in hybrid formation. Thus the concentration term $[C_f]$ used to calculate the rate constant may be incorrect and k_1 (hybridisation) may actually be equal to k_2 (reassociation). Alternatively, rate constants may be lower for filter hybridisation. As a consequence of binding nucleic acid sequences to the filter, steric restraints may retard the formation of stable nucleation complexes.

Equations 1 and 4 show that one of the factors affecting the kinetics of a filter hybridisation reaction is reassociation of the probe. This variable is often overlooked but its effects can be large and can cause problems in interpreting results. It has been shown that as much as 20 – 30% of the input DNA probe can be unavailable for hybridisation due to reassociation (9). A second complication is that the probe may form concatenates of partially-reassociated duplexes with single-stranded regions which can hybridise to filter-bound sequences. Again the effects are not negligible. Flavell *et al.* (11) showed that 10% of the added denatured, double-stranded DNA which hybridised to a filter containing single-stranded DNA represented homologous rather than complementary sequences. Similar problems can arise in DNA-RNA hybridisation experiments with self-complementary transcripts. In order to minimise these complications, it is desirable to choose reaction conditions which facilitate diffusion of probe to the filter and favour hybridisation over reassociation, that is, use of a small probe (preferably single-stranded), small reaction volume, a low concentration of probe in solution and a high reaction temperature.

3. FACTORS AFFECTING THE RATE OF FILTER HYBRIDISATION

3.1 Concentration of the Probe

There has been no systematic study of effects of the concentration of probe on the rate of hybridisation at the filter and on the yield of duplex. However, the following points should be noted.

3.1.1 *Double-stranded Probe in Excess*

If the probe is a simple double-stranded sequence, Equation 1 predicts that at high $[C_s]$ values, reassociation of the probe should be favoured over hybridisation to the bound nucleic acid. Therefore, as incubation continues, the reaction will change from being

in probe excess to being in filter-bound excess where, as we have seen, the kinetics are different. Increasing the concentration of probe in solution, $[C_s]$, will increase the initial rate of hybridisation at the filter and the proportion of filter-bound sequences in duplex will increase, but not dramatically. For DNA probes and filter-bound RNA, as in RNA dot blots, high concentrations of formamide can be used to suppress reassociation in solution (see Section 4.1.2).

3.1.2 *Single-stranded Probe*

Whether in excess or not, there is no reassociation of a single-stranded probe in solution unless there are regions of extensive self-complementarity. The rate of hybridisation to the filter and the amount of hybrid formed should increase with increase in $[C_s]$. It is important to note, however, that the probe concentration should not be increased without limit. If more than about 100 ng ^{32}P-labelled probe per ml is used, non-specific irreversible binding to the filter occurs.

3.2 Probe Complexity

For solution hybridisation, the rate of reassociation of DNA is an inverse function of its complexity, so that the more complex the DNA, the slower the rate of reassociation (2,12). Extending this to filter hybridisations, the rate of reassociation of the probe should fall when the complexity of the DNA increases and its effective $[C_s]$ decreases. This is indeed what is observed (9). In contrast, two effects of complexity are seen for hybridisation of the probe to filter-bound nucleic acid sequences. When $[C_f]$ is low, the rate of hybridisation is inversely proportional to complexity over a 400-fold range, indicating that the reaction is controlled by the nucleation step. However, when the hybridisation reaction is limited by diffusion of the probe to the filter, that is when $[C_f]$ is high, the rate of reaction is independent of complexity (9).

3.3 Molecular Weight of the Probe

For DNA-DNA hybridisation in solution, the rate is directly proportional to the square root of the molecular weight of the nucleic acid (12) and this also describes the reassociation of the probe in solution during filter hybridisation (9). However, the effect of the molecular weight of the probe on the rate of hybridisation to filter-bound sequences contrasts sharply with that found in solution. Two situations can occur. When $[C_f]$ is low compared with $[C_s]$, that is, a nucleation-limited reaction, the rate of hybridisation is independent of the molecular weight (9,10). When $[C_f]$ is high compared with $[C_s]$, that is, diffusion-limited filter hybridisation, the rate of hybridisation is inversely proportional to the molecular weight of the probe, but there are insufficient data for an exact relationship to be formulated. The observed rate of hybridisation is significantly depressed by an increase in the molecular weight of a single-stranded probe (which is not capable of reassociation). This effect is even more pronounced when a double-stranded probe is used. This is because the combined effects of a lower rate of hybridisation and the increased rate of reassociation, which accompanies an increase in molecular weight of the sequences in solution, result in lower observed rates of hybridisation and a reduced final yield of hybrid. The difference in dependence on molecular weight of the two types of filter hybridisation is not understood.

3.4 Base Composition

The base composition of nucleic acids affects the rate of hybridisation, the rate increasing with increasing % G+C. However, the effect is small (12) and can be ignored in practice.

3.5 Temperature

The temperature of reaction affects the rate of any hybridisation reaction (13). Typically a bell-shaped temperature dependence curve is obtained. At 0°C, hybridisation proceeds extremely slowly, but as the temperature is raised, the rate increases dramatically to reach a broad maximum which is 20−25°C below T_m for DNA-DNA annealing. At higher temperatures the duplex molecules tend to dissociate so that as the temperature approaches T_m −5°C, the rate is extremely low. The relationship applies to the formation of both well-matched and poorly-matched hybrids although the curve is displaced towards lower temperatures for mismatched duplexes (14). So, ideally, hybridisations should be carried out at a T_i (incubation temperature) that is 20−25°C below T_m. In practice, for well-matched hybrids, the hybridisation reaction is usually carried out at 68°C in aqueous solution and at 42°C for solutions containing 50% formamide. For poorly-matched hybrids, incubation is generally at 35 − 42°C in formamide-containing solutions.

A similar dependence has been shown for RNA-DNA hybridisations (10), but here the maximal rate of hybridisation is obtained at some 10−15°C below the T_m of the hybrids.

3.6 Formamide

Formamide decreases the T_m of nucleic acid hybrids (see Section 4.1). This is a very useful property because by including 30−50% formamide in the hybridisation solution, the incubation temperature, T_i, can be reduced to 30−42°C. This has several practical advantages: the probe is more stable at lower temperatures, there is better retention of non-covalently-bound nucleic acid on the filter and nitrocellulose filters are less likely to disintegrate at the lower temperature.

Concentrations of formamide between 30 and 50% apparently have no effect on the rate of filter hybridisation and 20% formamide reduces the rate by only about one-third (15). On the other hand, a concentration of 80% formamide is thought to depress the rate constant for hybridisation in solution at least by a factor of three for DNA-DNA duplexes and by a factor of 12 for RNA-DNA hybrids (16). Qualitatively similar results are likely to occur in filter hybridisation.

Formamide can be used to alter the stringency of the reaction conditions. By holding T_i constant and varying the concentration of formamide, different effective temperatures are obtained. Effective temperatures as low as 50°C below the T_m of perfectly-matched hybrids can be reached which allows detection of homologies with as much as 35% mismatching (15).

3.7 Ionic Strength

At low ionic strength, nucleic acids hybridise very slowly, but as the ionic strength increases, the reaction rate increases. The effect is most dramatic at low salt concen-

trations (<0.1 M Na^+) where a 2-fold increase in concentration increases the rate 5- to 10-fold. Above 0.1 M Na^+ the rate dependence is less, but still marked up to about 1.5 M Na^+ (12,17).

High salt concentrations stabilise mismatched duplexes, so to detect cross-hybridising species, the salt concentration of hybridisation and washing solutions must be kept fairly high. Washing is therefore generally carried out using 2 – 6 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0).

3.8 Dextran Sulphate

Wetmur (18) observed that the addition of an inert polymer such as dextran sulphate increased the rate of hybridisation in solution. Thus the presence of 10% dextran sulphate gave rise to a 10-fold increase in reassociation rate. The effect was attributed to the exclusion of the DNA from the volume occupied by the polymer, that is, the dextran sulphate effectively increased the concentration of the DNA. A qualitatively similar effect occurs in filter hybridisation using both DNA and RNA probes (19) where, of course, the concentrating effect of the polymer applies only to the solution phase. For a single-stranded probe, the rate of hybridisation increases by 3- to 4-fold. For a double-stranded probe, the rate apparently increases by up to 100-fold and the yield of hybrid apparently also increases. However, in both cases, most of this increase is caused by the formation of concatenates which readily occurs under these conditions, that is, extensive networks of reassociated probe which, by virtue of single-stranded regions, hybridise to filter-bound nucleic acid and so lead to over-estimation of the extent of hybridisation. For qualitative studies this amplification in the hybridisation signal caused by binding of labelled probe is quite useful. However, for quantitative studies the effect may complicate the interpretation of results. Therefore it may be desirable to reduce the likelihood of networks forming by using probes which are not self-complementary. If double-stranded DNA probes are used they should be short to minimise the formation of extensive networks of probe. For example, if nick-translated, double-stranded DNA probes (Chapter 2, Section 4.1.2) are used, the DNase concentration in the nick-translation reaction should be adjusted to give fragments $\leq$400 nucleotides long. Short incubation times should also be used since the formation of networks occurs late in the reaction. Finally it should be noted that solutions of dextran sulphate are viscous (and so can be difficult to handle) and can lead to high backgrounds.

3.9 Mismatching

Many hybridisation reactions involve complex mixtures of sequences and the duplexes formed are not all perfectly base-paired. Mismatching has the effect of lowering the rate of hybridisation and the melting temperature of hybrids, T_m. The temperature dependence of k_i, the rate constant for the formation of mismatched hybrids, still gives a bell-shaped curve (Section 3.5), but k_i is lower and reaches its optimum at a lower temperature relative to the rate constant for formation of perfect hybrids (4,14). This has not been studied extensively, but available data suggest that if the reaction is carried out at a temperature which is optimal for the formation of mismatched sequences, that is, about 25°C below their T_m, the rate is reduced by a factor of two for every 10% mismatch (14).

3.10 Viscosity

As the viscosity of the solution increases, the rate of hybridisation decreases. The effect can be quite large, but there is insufficient data to formulate an exact relationship.

3.11 pH

The effect of pH has not been studied extensively but within the pH range 5 – 9, the rate of hybridisation at 0.4 M Na^+ is essentially independent of pH (ref. 12). In practice, hybridisation experiments are usually carried out at pH 6.8 – pH 7.4.

4. FACTORS AFFECTING HYBRID STABILITY

The melting temperature T_m is a measure of the thermal stability of hybrids. No systematic study of the effect of different parameters has been made for filter hybridisation. However, in general, variables that alter the rate constant, k, also alter the T_m in the same direction. The relationships below are derived from studies on hybridisation in solution, but are expected to be similar, qualitatively at least, for filter hybridisation. It is worth noting, however, that as a consequence of binding nucleic acid to the filters, the T_m of hybrids is often lower than would be predicted from solution hybridisation studies (5).

4.1 Perfectly-matched Hybrids

4.1.1 *DNA-DNA Hybrids*

Many studies on the stability of perfectly-matched DNA duplexes in solution have shown that T_m is dependent on ionic strength, base composition and denaturing agents (14, 20,21). The following relationship has been derived from combining several results (15):

$$T_m = 81.5 + 16.6 \text{ (log M)} + 0.41 \text{ (\%G+C)} - 0.72 \text{ (\% formamide)} \qquad \text{Equation 7}$$

where M is the molarity of the monovalent cation and (% G+C) is the percentage of guanine and cytosine residues in the DNA. The monovalent cation dependence holds between the limits of 0.01 – 0.4 M NaCl, but only approximately above this (20). The T_m is maximal at 1.0 – 2.0 M NaCl. The dependence on (% G+C) is valid between 30% and 75% (G+C) (ref. 22). The reduction in T_m by formamide is greater for poly(dA:dT) (0.75°C per 1% formamide) than for poly(dG:dC) (0.5°C per 1% formamide) (ref. 16).

In aqueous solution at 1 M NaCl (equivalent to 6 x SSC), Equation 7 simplifies to:

$$T_m = 81.5 + 0.41 \text{ (\% G+C)}$$

The following relationships, derived from solution hybridisation studies, are also useful:

(i) Every 1% mismatching of bases in a DNA duplex reduces the T_m by 1°C (ref. 14).

(ii) $(T_m)\ \mu_2 - (T_m)\ \mu_1 = 18.5 \log \mu_2/\mu_1$
where μ_1 and μ_2 are the ionic strengths of the two solutions (ref. 23).

4.1.2 *RNA-DNA Hybrids*

For RNA-DNA hybrids, the term in Equation 7 incorporating formamide concentration does not hold because the relationship between formamide concentration and the depression of T_m is not linear. At 80% formamide, RNA-DNA hybrids are more stable than DNA-DNA hybrids by some 10−30°C depending on the sequence (5,16). Carrying out the reaction in 80% formamide can therefore also be used to suppress formation of DNA-DNA duplexes and preferentially select RNA-DNA hybrids (5,16,24).

4.2 Mismatched Hybrids

The T_m of nucleic acid hybrids is depressed by base mismatching. Values obtained from solution hybridisation studies show that a 1% mismatch reduces the T_m by between 0.5 and 1.4°C (refs. 17,21,22,25,26). The exact figure depends on the (G+C) content of the DNA. The stability of the hybrids also depends on the distribution of mismatched bases in the duplex. Thus if two sequences have 20% base pair mismatch, the hybrid formed between them will have a high T_m if the mismatch is concentrated in one region leaving a long stretch of perfectly-matched duplex. In contrast, the hybrid will be extremely unstable if every fifth base is mismatched.

At high concentrations of salt, mismatched hybrids are more stable than at low concentrations. In practice this is very useful because varying the salt concentration can be used to stabilise or dissociate mismatched hybrids according to the requirements of the experiment.

5. DISCRIMINATION BETWEEN RELATED SEQUENCES

5.1 Stringency of hybridisation

A sizeable fraction of the eukaryotic genome is composed of families of similar, but not identical, sequences. It is often the aim of filter hybridisation studies to distinguish between closely- and distantly-related members of such a family, for example, in screening recombinant libraries or determining gene copy numbers by Southern blots (Chapter 5). In practice this means that reaction conditions must be adjusted to optimise hybridisation of one species and minimise hybridisation of others.

As explained in Section 3.5, bell-shaped curves describe the relationship between the rate of hybridisation and the temperature of incubation for formation of both well-matched and poorly-matched hybrids. For a poorly-matched hybrid, the rate constant is lower and the curve is displaced towards lower temperatures. When the ratio of rate constants (discrimination ratio) for cross-hybridisation and for self-hybridisation is plotted against temperature of reaction, a sigmoidal curve is obtained (*Figure 1*). At low temperatures, the ratio is high while at higher temperatures (approaching T_m −20°C for perfectly-matched hybrids), the ratio approaches zero (4,14). Although the data are not extensive, Beltz *et al.* (4) have suggested that this curve is probably a member of a family of sigmoidal curves whose exact dependence on temperature depends on the degree of mismatching of the hybrids. The relationship is useful in that it predicts that it should be easier to distinguish between distantly-related sequences by incubating at low temperatures while it should be easier to distinguish closely-related sequences by hybridising at high temperatures.

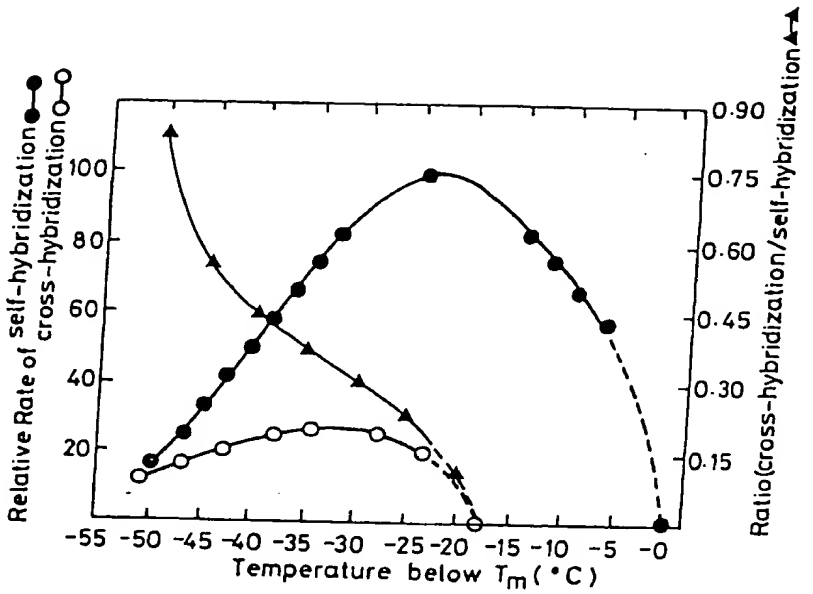

Figure 1. Rate of DNA reassociation as a function of temperature. Normal bacteriophage T4 DNA was used to examine the reassociation of perfectly-matched sequences (self-hybridisation; ●—●) and T4 DNA partially deaminated with nitrous acid was used for mismatched sequences (cross-hybridisation; O—O). The dotted lines are extrapolations assuming that the rates of reassociation are zero at the appropriate T_m: for normal T4 DNA under these conditions (0.15 M sodium phosphate buffer) the T_m is 81°C. The discrimination ratio (▲—▲) is the rate constant for cross-hybridisation k_i, divided by the rate constant for self-hybridisation, k. Reproduced from reference 4 with permission.

In practice, therefore, to distinguish between the distantly-related members of a family of sequences, hybridisation should take place at a very permissive (relaxed) criterion. To detect closely-related members, the hybridisation should be at a stringent criterion. A single compromise criterion will not be effective because, as we have seen, different members of the family probably have different discrimination *versus* temperature curves. Hybridisation at a relaxed criterion followed by washing under progressively more stringent conditions may be useful for detecting distantly-related members of a family, but is not suitable for identifying closely-related members. This is probably because hybridisation and washing depend on different parameters. Hybridisation depends on the nucleation frequency while washing depends on the thermal stability (T_m) of the hybrids. Thus, a stringent hybridisation followed by a stringent wash is better for detecting closely-related members of a family than permissive hybridisation and a stringent wash.

5.2 Extent of Reaction

In distinguishing between related sequences, it is important to consider the extent of reaction. At first sight, it might appear that the longer the time of incubation the better should be the discrimination, but this is not the case. The following arguments have been made by Beltz *et al.* (4).

When two (or more) filter-bound sequences react with the same probe, the rate of

depletion of the probe is given by the following equation:

$$\frac{-dC_s}{dt} = k_1[C_f][C_s] + k_2[C_s]^2 + k_i[C_i][C_s] \qquad \text{Equation 8}$$

where $[C_s]$ here refers to the concentration of the probe in solution at time zero.

This equation is derived from Equation 1 by the addition of a term to allow for cross-reaction of the probe with a related, filter-bound sequence, i, which has a concentration $[C_i]$ and a hybridisation rate constant k_i. $[C_f]$ is the concentration of filter-bound sequence, f, which is identical to the probe. The kinetics differ considerably depending upon which sequences are in excess and whether the probe can reassociate. For simplicity, in the following analyses (Sections 5.2.1 – 5.2.3) we have assumed that the concentrations of filter-bound sequences are the same (that is $[C_f] = [C_i]$) and $k_1 = k_2$. In fact we know that the rate constants are not equal, but the result will be qualitatively the same.

5.2.1 *Filter-bound Nucleic Acid in Excess*

When the filter-bound sequences are in excess over the probe, as in typical plasmid DNA dot blots (Section 6.2.1), Equation 8 simplifies to a pseudo-first order reaction where the rate of loss of the probe ($-dC_s/dt$) is given by:

$$\frac{-dC_s}{dt} = k_1[C_f][C_s] + k_i[C_i][C_s] \qquad \text{Equation 9}$$

Since $[C_f] = [C_i]$

then $$\frac{-dC_s}{dt} = [C_f]\,[C_s]\;\Sigma\, k$$

The rate of hybridisation to sequence i equals $[C_f][C_s]k_i$.
Therefore,

$$\frac{\text{rate of hybridisation to sequence i}}{\text{rate of hybridisation to all sequences}} = \frac{[C_f][C_s]k_i}{[C_f][C_s]\Sigma k} \qquad \text{Equation 10}$$

$$= \frac{k_i}{\Sigma k}$$

In a hybridisation reaction, it is more likely that there will be a number (m) of cross-reacting species. The overall reaction can be treated as the sum of a number of independent hybridisations each with a different rate constant and each following pseudo-first order kinetics. When they go to completion, the probe will all be in hybrids. The frac-

tion of the probe hybridised to sequence i is given by:

$$\frac{k_i}{\sum_1^m k}$$

At any time during the reaction, the ratio of the amounts of probe hybridised to sequence i and to any other filter-bound sequence j is given by the ratio of the rate constants (k_i/k_j) and the ratio is not affected by the time of incubation. Hence the discrimination between related hybrids is not affected by the extent of the reaction because all the filter-bound sequences continuously compete for the same limiting probe (*Figure 2*).

5.2.2 *Single-stranded Probe in Excess*

The kinetics of hybridisation are different from that described above when the probe is in excess over the filter-bound sequences, as in typical genomic Southern blots, genomic dot blots, RNA dot blots and Northern blots (Chapters 5 and 6). If the probe is single-stranded and so cannot reassociate (e.g., for M13 or SP6 RNA probes), Equation 8 simplifies to the same form as Equation 9:

$$\frac{-dC_s}{dt} = k_1[C_f][C_s] + k_i[C_i][C_s]$$

If $[C_f] = [C_i]$, then rearranging terms,

$$\frac{-dC_s}{dt} = [C_s] \sum_{i=1}^{i} k_i[C_i]$$

It can be shown that $E_i(t)$, the fraction of filter-bound sequence i actually hybridised at time t, is given by the equation:

$$E_i(t) = 1 - e^{-k_i[C_s]t} \qquad \text{Equation 11}$$

and the ratio of the extent of hybridisation of cross-hybridising sequence i, to perfectly-matched sequence f is:

$$\frac{E_i(t)}{E_f(t)} = \frac{1 - e^{-k_i[C_s]t}}{1 - e^{-k_1[C_s]t}} \qquad \text{Equation 12}$$

This ratio is not constant but varies with time. The discrimination (that is, the actual extent of cross-hybridisation compared with the hybridisation to perfectly-matched sequences) is maximal very early in the reaction when it equals k_i/k_1, but declines with increasing incubation time as the term at the right hand side of Equation 9 approaches unity (*Figure 2*). This means that although the homologous reaction is faster and will

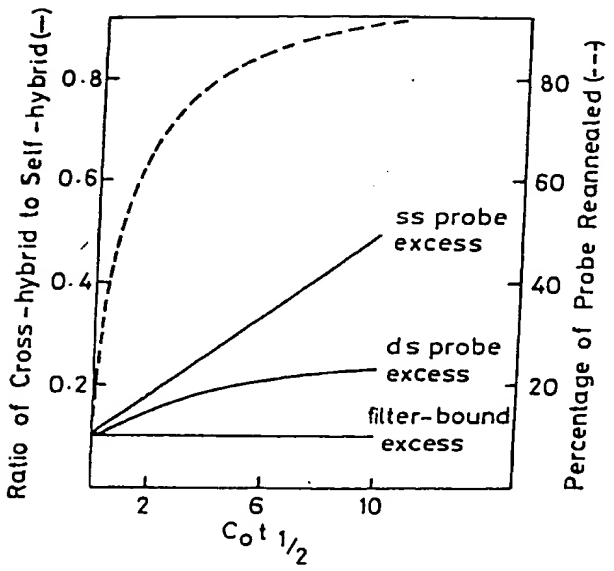

Figure 2. Effective discrimination between perfectly-matched and mismatched sequences as a function of the extent of the reaction. The solid lines represent the ratio [$E_i(t)/E_f(t)$, see text] of the amounts of probe hybridised to filter-bound mismatched, heterologous sequences (cross-hybridisation) and perfectly-matched, homologous sequences (self-hybridisation). Three separate reactions are shown: filter-bound sequences in excess; denatured double-stranded DNA probe in excess; single-stranded probe in excess. The discrimination ratio (k_i/k) is assumed to be the same (0.1) in all cases. The dashed line shows the normal kinetics of reassociation of denatured double-stranded DNA in solution. Reproduced from reference 4 with permission.

reach completion earlier, the heterologous reaction will eventually catch up (single-stranded probe excess; *Figure 2*). In practical terms, then, with increasing time of reaction, discrimination becomes poorer, so reaction times should be kept short.

5.2.3 *Double-stranded Probe in Excess*

Consider Equation 8 again:

$$\frac{-dC_s}{dt} = k_1[C_f][C_s] + k_2[C_s]^2 + k_i[C_i][C_s] \qquad \text{Equation 8}$$

If the probe can reassociate, the term $k_2[C_s]^2$ is significant. Therefore, as the reaction proceeds, reassociation in solution reduces the amount of probe that is available to hybridise to the filter-bound sequences. So the reaction changes from being in probe excess to one in which filter-bound nucleic acid is in excess. It can be shown that:

$$\frac{\text{fraction of probe hybridising to sequence i}}{\text{fraction of probe hybridising to perfectly-matched sequence f}} = \frac{E_i(t)}{E_f(t)}$$

$$= \frac{1 - (1+k_2[C_s]t)^{-n}}{1 - (1+k_2[C_s]t)^{-1}} \qquad \text{Equation 13}$$

where n = k_i/k_1, that is, the discrimination ratio. Again this means that in practical terms, discrimination equals k_i/k_1 very early in the reaction, but deteriorates rapidly. So, in practice, to distinguish between cross-hybridising species, it is best to use short times of incubation regardless of whether the probe or the filter-bound sequence is in excess. If this does not generate enough signal to be detected, it is advisable to use excess filter-bound sequence and to hybridise for longer times.

6. BINDING OF NUCLEIC ACID TO FILTERS

6.1 Types of Filter Material

There are several types of filter currently in use for the immobilisation of DNA and RNA, for example, nitrocellulose, nylon and chemically-activated papers. The material of choice depends on the purpose of the experiment.

Nitrocellulose filters bind DNA and RNA very efficiently, (~ 80 $\mu g/cm^2$) except for small fragments of less than about 500 nucleotides in length which are bound rather poorly. The binding procedure is simple. The main disadvantage of nitrocellulose is that it is rather fragile so it requires careful handling and on repeated use tends to become brittle and fall apart. Nylon filters are more pliable than nitrocellulose, are easier to handle and can be used indefinitely without disintegrating. They are reputed to bind nucleic acid as efficiently as nitrocellulose and on the whole we have found that this is true, but we have experienced batch variation in the binding efficiency of some brands. For Southern, Northern and dot blots, both nitrocellulose and nylon filters give excellent results. For DNA dot blots, filters with a pore size of 0.45 μm are used for large nucleic acid molecules and 0.22 μm for molecules of less than 500 nucleotides. For RNA dot blots, filters with 0.1 $-$ 0.22 μm pore size are most efficient.

Although nitrocellulose and nylon filters immobilise nucleic acid, binding by conventional procedures is not covalent. This can lead to problems. For example, nucleic acid is gradually leached off the surface when filters are hybridised for long periods, particularly at high temperature. Furthermore, if the probe in solution is complementary to the entire length of the filter-bound sequence, the hybrid dissociates from the filter and is lost into solution (27). So a consequence of non-covalent binding of nucleic acid is that the hybridisation sensitivity may be reduced with time. New techniques have now been developed for covalent binding of nucleic acid to membranes. This involves u.v. light-induced binding (28). However, to date this is only applicable to nylon filters because of the risk of fire when using nitrocellulose membranes. Chemically-activated paper binds nucleic acid covalently so it has the advantage that it does not discriminate against small nucleic acid molecules and does not lose nucleic acid sequences once they are bound. Both cellulose and nylon filters can be chemically activated. However, the binding capacity of chemically-activated paper (1 $-$ 2 $\mu g/cm^2$) is much lower than other types of filter and the binding procedure is more complicated. Hence it is not much used for Southern, Northern and dot blots; its main use is in hybrid selection for the enrichment of specific RNA sequences (Chapter 5, Section 3.2).

Suppliers of nitrocellulose filters are Schleicher and Schüll (membrane filters BA85), Millipore U.K. and Waters Associates (Millipore filters), Sartorius Instruments Ltd. (Sartorius filters) and Amersham International plc (Hybond C filters). Suppliers of nylon filters are New England Nuclear (GeneScreen and GeneScreen Plus hybridisation transfer

membrane), PALL (Biodyne transfer membrane) and Amersham International plc (Hybond-N membranes). Clearly, this list can never be complete since new products are continually being marketed. The filters are generally available as circles and rectangles in several sizes. Most are also available in rolls which can be cut to size.

All filters require the nucleic acid to be denatured for binding. However, it is most important to note that there is no immobilisation procedure uniformly applicable to all types of filter. Nitrocellulose filters require high ionic strength for quantitative binding of both DNA and RNA and the binding efficiency is much reduced at low ionic strength (29,30). In contrast, GeneScreen nylon membranes require low ionic strength for binding and the binding is poor at high salt concentrations. We have successfully used the same procedures for binding to nitrocellulose and to Biodyne nylon membranes.

6.2 DNA Dot Blots

Multiple samples of genomic or plasmid DNA are spotted next to each other on a single filter in dots of uniform diameter. For quantitative analysis, known amounts of DNA are applied. To evaluate the extent of hybridisation of the probe, a standard consisting of a dilution series of DNA dots is applied in an identical way to the same filter. The procedure binds samples quickly so that many samples can be handled at once. As little as 1 — 3 pg of a hybridising DNA sequence can be detected. Dot blots do not distinguish the number and size of the molecules hybridising, so the hybridisation 'signal' is the sum of all sequences hybridising to the probe under the conditions used.

Commercial apparatus has been developed for binding multiple samples of DNA to filters. A protocol for use of this is described elsewhere in this volume (Chapter 5, Section 3.1). Not every laboratory has access to such a device so the procedure described here involves manual application of samples. This is more time consuming and the dots are less uniform than when applied by the multiple filtration device, but the results are perfectly satisfactory.

There are many protocols in use for binding samples to filters. They can be divided into two classes according to whether the DNA is denatured before or after it is applied to the filter. Both give satisfactory results. An example of the former method is described for use with the multiple filtration device (Chapter 5, Section 3.1.2). We shall describe an example of the latter method which is in current use in our laboratory with nitrocellulose and Biodyne filters. It is not applicable to GeneScreen filters which require low salt concentration for DNA binding.

6.2.1 *Plasmid DNA Dot Blots*

It is necessary to convert supercoiled DNA to open circular or linear form to bind to filters. This is because DNA must be single-stranded for binding and denatured supercoiled DNA renatures too quickly on neutralisation to be trapped in the denatured state. Two common ways of obtaining linear or nicked plasmid DNA are to restrict the DNA by enzymic digestion and to treat the plasmid at high temperature (see *Table 1*). The latter partially depurinates the DNA so that on subsequent treatment with alkali the phosphodiester bond breaks at the site of depurination (31). Linear DNA will then separate into single strands.

Nitrocellulose filters are usually treated with high concentrations of salt either at the

Table 1. Linearisation of Plasmid DNA.

Restriction Method

1. Digest the recombinant plasmid with a suitable restriction enzyme. Monitor linearisation of the plasmid by agarose gel electrophoresis.
2. Extract the restricted DNA with an equal volume of phenol pre-saturated with 10 mM Tris-HCl. pH 7.5, 0.1 mM NaCl, 1 mM EDTA. Spin for 2 min in a microcentrifuge to separate the layers.
3. To the aqueous phase, add 0.5 vol. of 7.5 *M* ammonium acetate and precipitate the DNA by adding 2.5 vol. ethanol pre-cooled to −20°C. Mix well and place at −20°C overnight or −70°C for 1−2 h.
4. Recover the DNA by centrifugation and dry briefly under vacuum.
5. Resuspend the DNA at 50 μg/ml in TE buffer, pH 8.0[a].

High Temperature Method

1. Place 20 μg DNA in a microcentrifuge tube in a final volume of 100 μl 20 mM Tris-HCl, pH 7.4. 1 mM EDTA.
2. Pierce the lid to prevent it popping open and place the tube in a boiling water bath for 10 min.
3. Chill in ice and centrifuge for 10 sec to ensure that all the sample is at the bottom of the tube.
4. Check the volume and adjust to 100 μl with water if necessary so that the DNA concentration remains at 20 μg/100 μl.

[a]TE buffer is 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.

same time as, or prior to, binding of nucleic acid. This both improves the efficiency of binding and helps to keep the diameter of the dot small. Salts commonly used are 1 M ammonium acetate or 20 x SSC [1 x SSC is 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0]. A suitable procedure for binding plasmid DNA to nitrocellulose filters involving pre-treatment of the filter with high salt is given below. It is important to note that the filter must not at any stage be handled with bare hands. Grease from the fingers will result in poor binding of nucleic acid and high backgrounds. Therefore disposable plastic gloves must be worn at all stages.

(i) Float a sheet of nitrocellulose on water taking care not to trap air bubbles underneath. When one side is wet, immerse the filter completely to wet the other. If there are dry patches which are reluctant to wet, boil the water for a few minutes.

(ii) Blot the filter lightly on Whatman 3MM paper and transfer to a dish containing 20 x SSC. Leave for 30 min with gentle shaking.

(iii) Dry at room temperature or under a lamp until completely dry.

(iv) If convenient, use a conventional rubber stamp and ink pad to stamp the paper with an array of 5 mm diameter circles to allow easy identification of samples. At this stage, the filters can be stored dry at room temperature sealed in a polythene sleeve.

(v) Place a nitrocellulose filter which has been treated with 20 x SSC onto the lid of a plastic box such that only the edges of the filter are in contact with the lid.

(vi) Apply the plasmid (0.8 μg in 4 μl, linearised as in *Table 1*) to the filter. This can be achieved using a 1−5 μl Supracap pipette (Brand) or an automatic Pipetman (Gilson). Be careful not to puncture the nitrocellulose filter. Keep the diameter of the dots small and do not exceed 4 mm. If necessary, make repeated applications allowing time for each application to dry.

(vii) Allow the samples to dry at room temperature or under a lamp.

Table 2. Reducing the Size of Eukaryotic DNA Prior to Filter Binding.

1.	*Either* sonicate 30 μg DNA to an average size of 2 kb or restrict it with a suitable restriction enzyme and then remove the enzyme by phenol extraction (*Table 1*, step 2). The size of the DNA should be checked by agarose gel electrophoresis using appropriate size markers (Appendix II).
2.	Recover the DNA by ethanol precipitation (*Table 1*, step 3).
3.	Wash the DNA in 70% ethanol, dry briefly under vacuum and resuspend in 1 ml TE buffer, pH 8.0[a].
4.	Measure the concentration of DNA spectrophotometrically using the conversion factor $A_{260nm} = 1$ for a solution of 50 μg/ml.
5.	Check the volume of solution and freeze dry.
6.	Resuspend the DNA in water at a concentration of 10 μg per 4 μl. For serial dilutions, prepare a set of microcentrifuge tubes each containing 6 μl TE buffer, pH 8.0. Remove 6 μl DNA into the first tube containing TE buffer. Mix well. Remove 6 μl from this tube into the second tube with TE buffer and so on until seven dilutions have been made (10 μg−5.3 ng per 4 μl).
7.	Centrifuge briefly in a microcentrifuge to ensure that each DNA solution is at the bottom of the tube.
8.	Apply the samples to a dry sheet of nitrocellulose pre-treated with 20 x SSC as described in Section 6.2.1, steps (v) – (xii).

[a]TE buffer is 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.

(viii) Denature the DNA by placing the filter, application side up, on a sheet of Whatman 3MM paper saturated, but not 'swimming', in 1.5 M NaCl, 0.5 M NaOH. Leave for 5 min. (This is conveniently done in a plastic tray.)

(ix) Transfer the filter to Whatman 3MM paper saturated with 0.5 M Tris-HCl, pH 7.4, for 30 sec.

(x) Transfer the filter to Whatman 3MM paper saturated in 1.5 M NaCl, 0.5 M Tris-HCl, pH 7.4, for 5 min. The DNA is now reversibly bound to the filter.

(xi) Place the filter on a dry sheet of 3MM paper and leave to dry at room temperature.

(xii) Sandwich the filter between two sheets of 3MM paper and bake at 80°C for 2−3 h to immobilise the DNA. Ideally, nitrocellulose filters should be baked in a vacuum oven to reduce the risk of fire.

6.2.2 *Genomic DNA Blots*

Like plasmid DNA, genomic DNA can be applied to filters in dots. The amount of DNA required per dot depends on the experiment being performed. To detect a single copy sequence in eukaryotic DNA, a minimum of 10 μg DNA per dot is suggested. For sequences present in multiple copies, proportionately less can be used.

Modern techniques for DNA isolation usually give a product which is very concentrated and has a very high molecular weight. These two factors make the DNA solution very viscous so that it is difficult to measure the concentration accurately. Hence, to do this, it is necessary to reduce the size of the DNA either by sonication or digestion with an appropriate restriction endonuclease (*Table 2*). This also helps to bind the DNA to the filter more efficiently. Binding of the DNA to nitrocellulose filters, pretreated with 20 x SSC, is carried out as described above for plasmid DNA (Section 6.2.1).

6.3 RNA Dot Blots

The principle of this procedure is exactly the same as for DNA dot blots. Known amounts of RNA are applied to an inert support and the amount of specific RNA sequence is

determined by hybridisation with a suitable labelled probe. Evaluation of the extent of hybridisation can be made by comparison with standards. The technique is sensitive — as little as 1 pg of a specific RNA sequence can be detected (32). As with DNA dot blots, however, the procedure gives no information on the size or number of sequences contributing to the hybridisation signal. Nylon and nitrocellulose filters are suitable supports. Because nitrocellulose tends to be the most used, its use will be described here. Chemically-activated paper is not generally used for RNA dot blots as its binding capacity is too low.

6.3.1 *Preliminary Precautions*

One of the main problems of working with RNA is its extreme sensitivity to degradation. Glassware must be scrupulously clean and should never be touched with bare hands which are a good source of ribonuclease. Prior to use, the glassware should be treated with diethylpyrocarbonate to inactivate any ribonuclease. This can be done by immersing the glassware in water to which has been added two drops per litre of diethylpyrocarbonate and then boiling for 30 min. The glassware can then be dried. Heavy metal ions can lead to degradation of RNA especially when long incubations are involved so these should be removed by filtration of all solutions through Chelex resin (BioRad) before use.

6.3.2 *Denaturation of RNA and Binding to Filters*

Although RNA is single-stranded, it contains double-stranded regions which must be denatured for efficient binding to filters. Alkali treatment is not suitable since it degrades RNA and heat denaturation has not been found to give efficient binding (32). Commonly used denaturants for RNA include glyoxal (33), methyl mercuric hydroxide (34), formaldehyde (35) and dimethyl sulphoxide (DMSO) (36). Since methyl mercuric hydroxide and formaldehyde are toxic and DMSO dissolves nitrocellulose, the procedure described here uses glyoxal as a denaturant. It is based on the methods developed by Thomas (32,37). Glyoxal is supplied commercially as a 40% aqueous solution (6.89 M) which contains polymerisation inhibitors. Glyoxal is readily oxidised to glyoxilic acid which degrades RNA so it is necessary to purify the glyoxal before treating the RNA. This is usually done by deionisation. A suitable protocol is as described in Chapter 6, *Table 1*.

Glyoxal denatures RNA (and DNA) by binding covalently to guanine residues forming an adduct which is stable at acid and neutral pHs. Glyoxylated nucleic acid binds efficiently to nitrocellulose paper but, after binding, the glyoxal groups must be removed because they have an inhibitory effect on hybridisation. This is easily and quantitatively achieved by treating the filter at 100°C at pH 8.0. Under these conditions as little as 1 pg of a specific sequence of RNA can be detected. The detailed procedure for glyoxalation of RNA and binding to nitrocellulose filters is as follows.

(i) Dry down 20 μg of the sample RNA in a microcentrifuge tube and dissolve it in 5 μl water. The RNA should be salt-free and free of protein which will otherwise react with the glyoxal.

(ii) Prepare a denaturation solution: 34 μl deionised glyoxal, 20 μl 0.1 *M* sodium phosphate buffer, pH 6.5, 46 μl water.

(iii) Add 5 μl denaturation solution to the RNA. Cover the tube and incubate for 1 h at 50°C. (If using a water bath, make sure that the water comes well up the sides of the tube in order to minimise evaporation.)

(iv) Centrifuge in a microcentrifuge (10 sec) to ensure the sample is at the bottom of the tube. After denaturation in glyoxal, the samples are stable for a few hours and can be kept at 4°C.

(v) If required, make serial dilutions as described in *Table 2* (step 6) but using 1% SDS as the diluent.

(vi) Apply the samples by hand to a sheet of nitrocellulose [pre-treated in 20 x SSC as described in Section 6.2.1, steps (i)—(iv)] using the application procedure described in Section 6.2.1, steps (v) and (vi).

(vii) Bake the filter at 80°C for 2 h to immobilise the RNA.

(viii) Remove the glyoxal groups by placing the filter in water at 100°C for 5—10 min, then allowing the water to cool to room temperature.

(ix) Blot the filter on 3MM paper and allow to dry at room temperature or under an infra-red lamp.

7. NUCLEIC ACID PROBES

7.1 Types of Probe

In theory, any nucleic acid can be used as a probe provided that it can be labelled with a marker which allows identification and quantitation of the hybrids formed. In practice, double- and single-stranded DNAs, mRNA and RNAs synthesised *in vitro* are all used as probes. Oligonucleotide probes are not used in quantitative dot blots; they are most useful for screening recombinant DNA libraries.

7.1.1 *Double-stranded DNA Probes*

Double-stranded DNA probes are very commonly used in dot blot analysis. They are often cloned sequences and have low complexity. There are two important points to note when using double-stranded DNA probes:

(i) Two competing reactions occur in filter hybridisation, *viz.* reassociation of the probe in solution and hybridisation to the filter-bound nucleic acid. Therefore, reaction conditions should be chosen to optimise the latter (see Section 11).

(ii) If the DNA is a cloned sequence, it should be excised and purified away from the vector. This avoids complications which can arise if single-stranded vector tails allow formation of concatenates in solution, particularly if the DNA has been randomly sheared. Furthermore, if filters are re-hybridised and the previous probe containing vector sequences has not been completely removed, sandwich hybridisation may occur. That is, duplexes may form through vector sequences rather than through insert sequences. This complicates the interpretation of results.

7.1.2 *Single-stranded DNA Probes*

With single-stranded DNA probes there is no competing reassociation in solution so filter hybridisation is favoured and reactions can be carried out for longer. Single-stranded DNA probes are obtained by strand separation of double-stranded DNA (see Chapter 6, Section 4.2.4) or from M13 phage recombinants.

7.1.3 *RNA Probes*

RNA probes are more difficult to handle than DNA probes because of the widespread presence of ribonucleases. In addition, mRNA probes, or cDNAs derived from them, are often complex mixtures of sequences and therefore the sequence of interest may represent only a very small proportion of the total nucleic acid. Since the rate of filter hybridisation is inversely proportional to the complexity for low amounts of filter-bound nucleic acid, it may be difficult or impossible to detect the desired hybrids.

Recently it has proved possible to synthesise large amounts of RNA *in vitro* from specially-constructed recombinant plasmids, such as the SP6 plasmids. The probes have low complexity and, because they are single-stranded, there is no competing reassociation reaction in solution. For these reasons, the use of SP6 RNA transcripts as probes is proving increasingly popular.

7.2 Radiolabelled Probes

Traditionally, filter hybridisations have been carried out with radioactively-labelled probes. ^{32}P is the most commonly used radionuclide and will be the only one discussed here. Conventional labelling replaces a proportion of the nucleotides in a nucleic acid with ^{32}P derivatives or adds ^{32}P to the end of the molecule. After hybridisation, hybrids are detected by autoradiography. ^{32}P has the advantage over other radioisotopes that high specific activities can be readily attained. Much of the technology of filter hybridisation has been developed with it. However, precautions must be taken when handling ^{32}P because of the radiation emitted. Detection by autoradiography, while sensitive, may take a long time if there are few counts in the hybrids. Furthermore, since ^{32}P has a half-life of 14.3 days, experiments should be completed within one half-life.

The preparation of radioactively-labelled DNA and RNA probes, including SP6 RNA transcripts, is described in detail in Chapter 2. In preparing labelled probes for filter hybridisations it is important to remove unincorporated precursors efficiently before use (see Chapter 2) otherwise they may bind non-specifically, but irreversibly, to the filter, giving a high background.

7.3 Non-radioactive Probes

Recent advances in nucleic acid technology now offer alternatives to radioactively-labelled probes. For example, single-stranded DNA can be coupled to a protein. If this protein-DNA complex is now hybridised to filter-bound nucleic acid, the protein in the duplex can be visualised by an antibody reaction (38). If the protein is an enzyme such as peroxidase, then it can be detected and quantitated by its ability to convert a colourless substrate into an insoluble coloured pigment at the site of hybrid formation. This technique is sensitive (1 — 5 pg nucleic acid can be detected) and has some potentially useful applications. For example, DNA probes coupled to different enzymes can be used in the same hybridisation reaction, so that it should be possible to detect the presence of unrelated sequences simultaneously (38).

Another procedure that uses non-radioactive probes and is becoming increasingly popular is biotin labelling of nucleic acid (39,40). These probes are prepared in a nick-translation reaction by replacing nucleotides with biotinylated derivatives. After hybridisation and washing, detection of hybrids is by a series of cytochemical reactions which

finally give a blue colour whose intensity is proportional to the amount of biotin in the hybrid. Biotinylated probes detect target sequences with the same sensitivity as radioactive probes, that is, in the 1 – 5 pg range. There are several advantages of using biotinylated probes. For example, non-toxic materials are employed and there are no problems of inconveniently short half-lives of the label. This has the additional bonus that biotin-labelled probes can be prepared in advance in bulk and stored at −20°C until required. Detection of hybrids is much faster than for radioactive probes, visualisation of hybrids being complete 2 – 4 h after washing. One disadvantage of biotin-labelled probes is that the cytochemical visualisation reactions lead to precipitation of insoluble material which cannot be removed, so when the filter is re-used, the previous 'signals' are still present (39,40). The preparation and use of non-radioactive nucleic acid probes is discussed in Chapter 2, Section 4.3.

7.4 Additional Considerations

Additional factors which should be borne in mind when choosing probes are:

(i) It is important to characterise the nucleic acid used for the probe. If any repetitive elements are present (e.g., *Alu*I sequences), they must be removed if the probe is to be used to detect low copy number sequences otherwise hybridisation of the latter will be masked by the repetitive sequence hybridisation.

(ii) The length of the labelled probe is important since the kinetics of hybridisation depend on probe length (see Section 3.3).

(iii) The kinetics of hybridisation differ according to whether the probe or filter-bound sequences are in excess and it is not always immediately apparent which is in excess. What is important is the concentration of the hybridising species, not the total nucleic acid concentration. The following is a rough guideline to this problem. With genomic Southern and Northern blots and genomic DNA and RNA dot blots, the concentration of the probe is likely to be in excess. For example, in *Figure 3*, even though there is 10 μg RNA per dot and the Ha-*ras*1 probe is at 20 ng/ml, the probe is in excess for RNA taken from normal tissue. (However, note that the filter-bound sequences are in excess for RNA taken from diseased tissue.) With plasmid or phage dot blots, and phage and colony screening, the filter-bound sequences are likely to be in excess. However, to check which is in excess, the following preliminary experiments can be performed.

(a) Vary the input of probe; if the filter-bound sequence is in excess, the amount of hybridisation should be proportional to the probe input.

(b) Vary the amount of nucleic acid on the filter; if it is in excess, there should be no difference in the amount of probe hybridised.

8. HYBRIDISATION USING RADIOACTIVE PROBES

8.1 Choice of Reaction Conditions

There are many protocols available for hybridising a probe in solution to nucleic acid immobilised on filters. The conditions used depend on the purpose of the experiment and in general are governed by whether DNA-DNA or DNA-RNA hybridisation is involved and whether closely-related or distantly-related sequences are reacting. Reaction conditions that permit formation of hybrids which have a high degree of mismat-

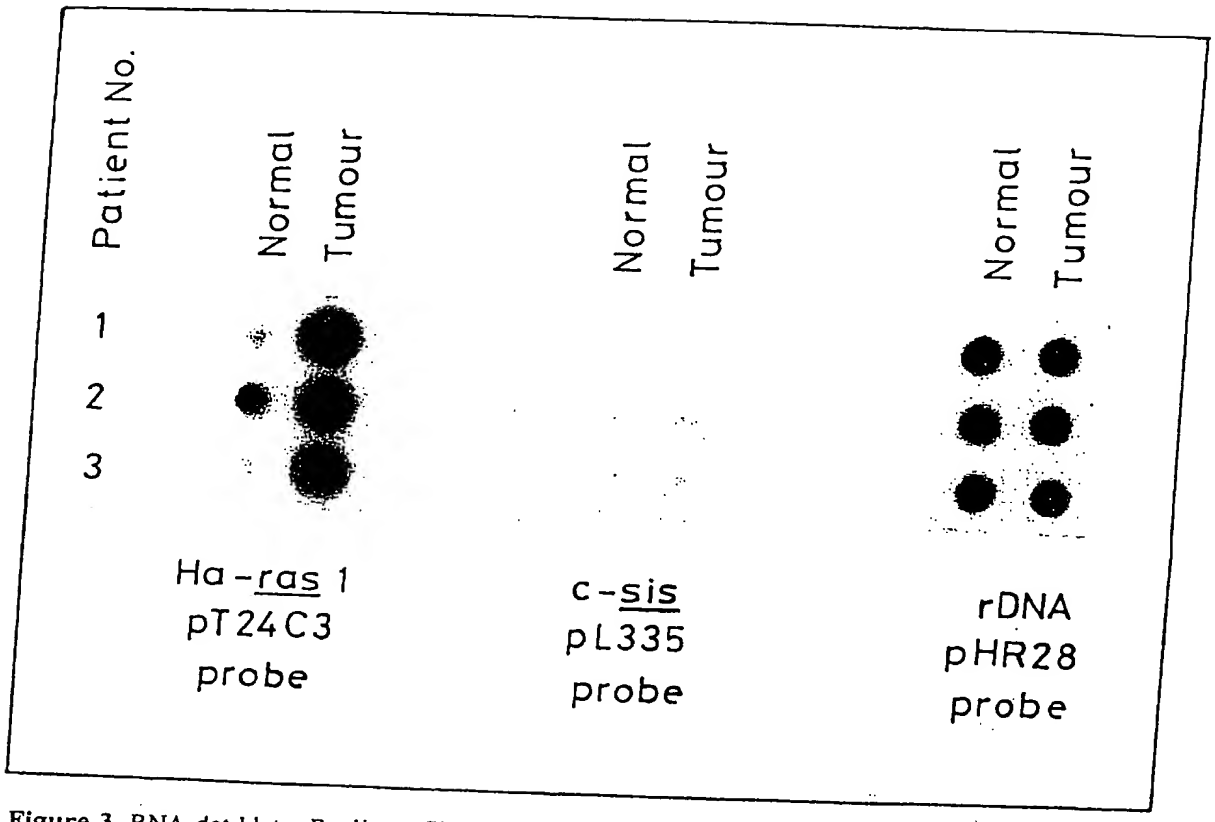

Figure 3. RNA dot blots. Replicate filters containing 10 μg per dot of poly(A)$^+$ RNA from normal and tumour breast tissue of three different patients were hybridised with the following denatured ^{32}P-labelled double-stranded probes: pT24C3 (Ha-*ras*-1), pL335 (c-*sis*) and pHR28 (rDNA). Equal amounts of RNA were present in the dots as judged by the intensities of the hybridisation signals using the rDNA probe. Transcripts homologous to the Ha-*ras*-1 probe are more abundant in tumour tissue compared with normal breast tissue and this difference is specific to the *ras* oncogene since the c-*sis* probe did not give a signal. (Data of Agnatis and Spandidos, with permission.)

ching are said to be permissive (relaxed or low stringency), while those which allow only well-matched hybrids to form are said to be stringent (high stringency). However, the same basic procedure is followed irrespective of the particular reaction conditions used. We will first describe standard hybridisation protocols which have widespread applicability (Sections 8–10) and then how varying the reaction conditions can be exploited to detect different hybrids (Section 11).

The hybridisation process can be divided into three steps: pre-hybridisation, hybridisation with a labelled single-stranded probe, and washing. In the *pre-hybridisation* step, the filter is incubated in a solution which is designed to pre-coat all the sites on it which would bind the probe non-specifically. Failure to do this leads to high backgrounds. Typically, the solution contains Ficoll, polyvinyl pyrollidone and bovine serum albumin [i.e. Denhardt's solution (ref. 41)], and heterologous DNA. As an alternative, heparin can substitute for Denhardt's solution (42). To reduce backgrounds even further, poly(A) and poly(C) are often included. Poly(A) is useful when the probe or filter-bound sequences are rich in A and T residues, e.g., poly(A)+ mRNA or cDNA derived from it. Similarly, poly(C) is included if the probe or filter-bound sequences are rich in G and C residues as when a recombinant is generated through oligo(dG) and oligo(dC)

homopolymer tailing. For hybridisations involving RNA, yeast tRNA is often used as a competitor. For *hybridisation*, it is necessary to ensure that the added nucleic acid probe is single-stranded. For double-stranded DNA probes this is usually achieved by boiling or by denaturing in alkali. Radioactive probes can be denatured using either method; heat denaturation is described here and alkaline denaturation is described in Chapter 5 (*Table 3*, step 6). For most purposes hybridisation can be carried out in either aqueous solution or in the presence of formamide. We use the same formamide-containing solutions for both RNA-DNA and DNA-DNA hybridisations, but aqueous solutions for DNA-DNA hybridisations only. Both protocols can be used with nitrocellulose and nylon filters and are described below. After hybridisation, *washing* is carried out to remove unhybridised probe and to dissociate unstable hybrids. The temperature and salt concentration of the washing solution determine which hybrids will be dissociated. In general, washing should be under as stringent conditions as possible; at 5 – 20°C below T_m for well-matched hybrids and 12 – 20°C below T_m for cross-hybridising species. In practice, 65 – 70°C is usually chosen for hybrids having a high degree of homology and 50 – 60°C for poorly-matched hybrids.

Where possible, the pre-hybridisation, hybridisation and washing steps should be carried out in a shaking water bath or on a shaking platform in an incubator. In filter-bound nucleic acid excess, diffusion of the probe to the filter can be limiting in the absence of agitation. Also, high backgrounds are sometimes encountered if there is no shaking. Solutions should be pre-warmed to the required temperature prior to use.

8.2 Hybridisation in the Presence of Formamide

8.2.1 *Pre-hybridisation*

(i) To wet the filters evenly, float them on a solution of 1% Triton X-100, taking care to prevent air bubbles being trapped underneath. When one side is wet, immerse the filter to wet the other side.

(ii) Remove the filters and blot gently on Whatman 3MM paper to remove excess liquid.

(iii) It is convenient to carry out the pre-hybridisation and hybridisation reactions in the same container. Typically this is a polythene bag. Suitable bags are 'Sears Seal-N-Save Boilable Cooking Pouches' or Layflat polythene tubing (Trans-Atlantic Supplies). Place each wet filter in a separate bag and heat-seal this, except for one corner, using a domestic bag sealer.

(iv) Add the pre-hybridisation solution (0.08 ml/cm^2 of filter) which has been prepared as described in *Table 3* and pre-warmed to 42°C. Gently squeeze out the air bubbles and heat seal the corner.

(v) Incubate the filter in the bag for 4 – 24 h at 42°C. This can be done by placing the bag in a box of water at 42°C in a shaking water bath at the same temperature. Set the water bath to shake at a speed such that the liquid in the bag sweeps gently over the surface of the filter. Alternatively, place the bag on a shaking platform in an incubator at 42°C.

(vi) Cut a corner of the bag and drain the liquid out. Roll a pipette over the surface of the bag to remove as much of the liquid as possible. However, it is most

Table 3. Preparation of Pre-hybridisation Buffer Containing Formamide.

Solution A	
Mix together:	
Deionised formamide[a]	50 ml
20 x SSC[b]	25 ml
100 x Denhardt's solution[c]	5 ml
1 M sodium phosphate buffer, pH 6.8[d]	5 ml
20% SDS[e]	0.5 ml
Adjust the volume to 95.5 ml with water.	
Solution B	
Mix together:	
Sonicated calf thymus DNA or salmon sperm DNA at 5 mg/ml[f]	2 ml
Poly(C) [5 mg/ml][g]	0.2 ml
Poly(A) [5 mg/ml][g]	0.2 ml
Yeast tRNA (5 mg/ml)[g]	2 ml
Denature in a boiling water bath for 5 min.	
Quench in ice.	
Add solution B to solution A and store at 4°C.	

[a]Formamide is a teratogen. Handle with care and use gloves. All contaminated glassware should be soaked overnight in dilute H_2SO_4 then rinsed with water before washing as usual. To deionise formamide, add 200 ml formamide to ~10 g of AG501-X8(D) mixed-bed resin (Bio-Rad). Stir for 1 h at room temperature. Filter through Whatman No. 1 filter paper to remove the resin. Store at 4°C in a dark bottle.
[b]The composition of SSC is 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0.
[c]100 x Denhardt's solution contains 2% Ficoll (mol. wt. 400 000), 2% polyvinyl pyrrolidone (mol. wt. 400 000) and 2% bovine serum albumin. Store at −20°C.
[d]1 M sodium phosphate buffer, pH 6.8, is made by mixing 25.5 ml of 1 M NaH_2PO_4 and 24.5 ml of 1 M Na_2HPO_4. Store at room temperature.
[e]Store this stock solution at room temperature.
[f]Add the DNA to water at ~5 mg/ml. Stir; it may take several hours for the DNA to dissolve. Then sonicate to a length of 400−800 bp. The size can be checked by agarose gel electrophoresis. Adjust the concentration to 5 mg/ml [A_{260nm} = 1 for a solution of 50 μg/ml]. Store at −20°C.
[g]These solutions are stored at −20°C. Their addition to the pre-hybridisation buffer is optional (see Section 8.1). However, there is no disadvantage in adding them even if T- and G-rich sequences are not present in the filter-bound nucleic acid sequence.

important that the filter is not allowed to dry out if high backgrounds are to be avoided. Therefore the filters should be left in the pre-hybridisation buffer in the bag until just before applying the hybridisation solution.

8.2.2 *Hybridisation*

(i) Except for single-stranded probes such as RNA and M13 probes, denature the labelled probe by placing it in a boiling water bath for 5 min. Quench in ice.

(ii) The hybridisation can be carried out in the presence of dextran sulphate which increases the rate of hybridisation (Section 3.8) or in its absence. For hybridisation buffer containing dextran sulphate (prepared as described in *Table 4*), pre-warm the buffer and add the denatured probe to a concentration which does not exceed 10 ng probe/ml or high backgrounds may ensue. In the absence of dextran sulphate, the probe concentration can be increased to 50−100 ng (43). For radioactive probes which have been labelled to a specific activity of

Table 4. Preparation of Standard Hybridisation Solution Containing Formamide.

Solution A	
Mix together:	
Deionised formamide[a]	50 ml
20 x SSC[b]	25 ml
100 x Denhardt's solution[c]	1 ml
1 M sodium phosphate buffer, pH 6.8[d]	2 ml
20% SDS[e]	1 ml
Dextran sulphate (mol. wt. 500 000)[h]	10 g
Stir until the dextran sulphate has dissolved. Adjust the volume to 95.5 ml.	
Solution B	
Mix together.	
Sonicated DNA (5 mg/ml)[f]	2 ml
Poly(C) [5 mg/ml][g]	0.2 ml
Poly(A) [5 mg/ml][g]	0.2 ml
Yeast tRNA (5 mg/ml)[g]	2 ml
Denature in a boiling water bath for 5 min.	
Quench in ice.	
Add solution B to solution A and store at 4°C.	

[a–g]See corresponding footnotes to *Table 3*.
[h]The inclusion of dextran sulphate is optional (see text, Section 3.8).

1–2 x 10^8 c.p.m./μg, a probe concentration of 10 ng/ml gives a solution of about 1–2 x 10^6 c.p.m./ml).

(iii) Immediately add this solution to the filter (0.05 ml/cm^2 filter) and reseal the bag.

(iv) Hybridise the filter at 42°C for the required time. This is normally between 6 and 48 h. Overnight is convenient and for many purposes is sufficient, but see Section 11.2.

8.2.3 *Washing*

(i) Cut one corner from the bag and remove the hybridisation solution. Retain the probe if it is to be re-used (see Section 13), otherwise discard it down a designated sink.

(ii) Cut open the bag completely and immerse the filter in 200 ml of 2 x SSC, 0.1% SDS at room temperature. Shake the filter gently. Rinse the filter twice for 5 min each time in this solution.

(iii) For a moderately stringent wash, wash the filter twice in 400 ml of 2 x SSC. 0.1% SDS at 60°C for 1 h. For a higher stringency wash, treat the filter for 2 x 1 h at 65°C in 0.1 x SSC, 0.1% SDS.

(iv) Finally, rinse the filter in 2 x SSC at room temperature. Blot the filter to remove excess liquid but do *not* dry the filter if it is to be re-washed or re-screened (Section 13).

(v) Detect the hybrids as described in Section 10.1.

8.2.4 *Washing with Nuclease Treatment*

In principle, blots can be treated with nucleases to remove unpaired loops and single-

stranded probe tails. This practice should increase the specificity of the reaction, but it has not been studied systematically and there is no real evidence that the treatment is effective. It is better to control specificity by careful choice of reaction and dissociation conditions than through enzyme digestion. DNase and nuclease S1 treatments are not generally used in filter hybridisations, but RNase treatment occasionally is. Filters are treated with a mixture of RNase A and T1 RNase at 25 μg/ml and 10 units/ml, respectively, in 2 x SSC at 37°C for 2 h, then washed in 2 x SSC, 0.5% SDS at 68°C, and finally in 2 x SSC at room temperature.

8.3 Hybridisation in Aqueous Solution

8.3.1 *Pre-hybridisation*

(i) Wet the filter in 1% Triton X-100 and blot to remove excess liquid.

(ii) Immerse in 4 x SET buffer for 15 min at room temperature. The composition of 4 x SET buffer is 0.6 M NaCl, 1 mM EDTA, 80 mM Tris-HCl, pH 7.8.

(iii) Transfer the wet filter to a plastic bag and heat seal this except for one corner (see Section 8.2.1). Add pre-hybridisation buffer (0.08 ml/cm² of filter), prepared as described in *Table 5* and pre-warmed to 68°C. Incubate at 68°C for between 2 and 16 h.

(iv) Open the bag and remove the pre-hybridisation buffer (see Section 8.2.1 for methodology).

8.3.2 *Hybridisation*

(i) Denature the probe as described in Section 8.2.2 and add it to fresh pre-hybridisation buffer (pre-warmed to 68°C) at 10–25 ng/ml. Add this solution to the filter at 0.05 ml/ cm² of filter.

(ii) Incubate at 68°C for between 5 and 16 h.

Table 5. Preparation of Aqueous Pre-hybridisation Buffer.

Solution 1	
Mix together:	
20 x SET buffer[a]	20 ml
100 x Denhardt's solution[b]	10 ml
20% SDS[b]	0.5 ml
5% sodium pyrophosphate	0.1 ml
Adjust the volume to 97.5 ml with water.	
Solution 2	
Mix together:	
Sonicated DNA (5 mg/ml)[b]	2 ml
Poly(C) [5 mg/ml][b]	0.2 ml
Poly(A) [5 mg/ml][b]	0.2 ml
Denature in a boiling water bath for 5 min.	
Quench in ice.	
Add to solution 2 to solution 1 and store at 4°C.	

[a] 1 x SET = 0.15 M NaCl, 20 mM Tris-HCl, pH 7.8, 1 mM EDTA.
[b] See relevant footnote to *Table 3*.

8.3.3 *Washing*

(i) Cut one corner from the bag and remove the hybridisation solution. Retain the probe if it is to be re-used (see Section 13), otherwise discard it down a designated sink.

(ii) Cut open the bag completely. Remove the filter and immerse it in 4 x SET buffer, 0.1% SDS, 0.1% sodium pyrophosphate for 5 min at room temperature.

(iii) Wash the filter three times in 2 x SET, 0.1% SDS, 0.1% sodium pyrophosphate for 20 min each wash at 68°C.

(iv) For a moderately stringent wash, wash the filter three times in 1 x SET, 0.1% SDS, 0.1% sodium pyrophosphate for 20 min each wash at 68°C. For a higher stringency wash, replace the 2 x SET by 0.1 x SET.

(v) Rinse the filter for 5 min in 2 x SET, at room temperature.

(vi) Blot the filter to remove excess liquid but do *not* dry the filter if it is to be re-washed or re-screened (Section 13).

(vii) Detect the hybrids as described in Section 10.1.

9. HYBRIDISATION USING BIOTIN-LABELLED PROBES

The hybridisation procedure for biotin-labelled probes is essentially the same as for radioactively-labelled probes (Section 8) except that the following points should be noted (39,40):

(i) The probe should be denatured at high temperature and not with alkali because the amide bond in the linker molecule between the biotin and nucleic acid is alkali-labile.

(ii) Hybridisation is carried out in solutions containing formamide rather than at high temperature. The thermal stability of biotin-labelled hybrids is slightly lower than that of radioactive hybrids. So, in practice, the formamide concentration is lowered from 50% to 45% in otherwise standard hybridisation conditions.

(iii) Certain types of polythene bags are not suitable for hybridisation with biotin-labelled probes as they lead to high backgrounds. Layflat polythene tubing (Trans Atlantic Supplies) and Sears' Boilable Cooking Pouches are both suitable.

(iv) Since very low background signals are obtained with biotinylated probes, the concentration of probe can be increased to 250 – 750 ng/ml in the hybridisation solution. This has the additional advantage of allowing short hybridisation times of 1 – 2 h.

10. DETECTION AND QUANTITATION OF HYBRIDS

The detection of hybrids involving probes labelled with non-radioactive markers is described in Chapter 2, Section 4.3. Here we shall consider only radioactive hybrids.

10.1 Detection

For detecting [^{32}P]hybrids, autoradiography is the most commonly used technique. It is sensitive, gives good resolution and does not involve destruction of the filter.

If the filter is not to be re-screened or re-used, dry it at room temperature or under an infra-red lamp. Then expose it to X-ray film (e.g., Kodak X-Omat RP) at room

temperature in a light-proof cassette. The time of exposure will vary from several hours to 14 days depending on the level of radioactivity in the hybrids. As a rough guide, a dot containing 100 c.p.m. ^{32}P will give a good signal on X-ray film with an overnight exposure. If the radioactivity levels are low, use of an intensifying screen (e.g., Ilford fast tungstate or Fuji Mach II) increases the sensitivity of the film by 4- to 5-fold. The film is sandwiched between the filter and the intensifying screen in the cassette. Exposure is at −70°C because fluorescence reflected off the intensifying screen is prolonged at low temperatures. If two intensifying screens are used, the sensitivity of the film is enhanced 8 − 10 times. In this case the filter and film are sandwiched between the two intensifying screens. For low levels of radioactivity, the film can be pre-flashed and placed flashed side against the intensifying screen with the filter on top (Chapter 5, *Table 3*, step 15). As few as 5 − 10 c.p.m. per dot above background can be evaluated dependably by this adaptation of autoradiography.

If the filter is to be re-used (Section 13.1), it must not be allowed to dry otherwise the probe will bind irreversibly. The wet filter is covered in Clingfilm or Saranwrap or is inserted into a thin polythene sleeve before exposure to X-ray film. The filter should not be too wet or ice crystals will form when the cassette is placed at −70°C. This will distort the filter and could cause it to crack.

10.2 Quantitation of Hybridisation Signals

For many purposes it is sufficient to compare visually the intensity of hybridisation signal on an autoradiogram with that generated by a standard series of dots. The accuracy is better than 2-fold over a 100-fold range, taking into account both the intensity and diameter of the autoradiographic spots. For example, *Figure 4* shows dot blots of genomic DNA from patients suffering from chronic myeloid leukaemia (CML) and from two CML cell lines, K562 and NALM-1, probed with the c-*sis* and c-*abl* oncogenes and a human immunoglobulin λ light chain variable gene sequence (IgV_λ). Visual comparison of the autoradiographic signals indicates that cell line K562 contains about four times more copies of the c-*abl* and IgV_λ genes than the other cell lines.

For more accurate quantitation, densitometry can be used. This is a very simple and sensitive procedure; as little as 5 − 10 c.p.m. above background can be evaluated reliably. It is the best method of quantitation when the amount of radioactivity in hybrids is low. A scan is made of a series of standard dots and of the unknown samples. The area under the peaks is integrated, either electronically or the peaks can be cut from paper traces and weighed. The weight of the paper is a measure of the autoradiographic signal. A graph is then plotted of the weight of (or area under) the standard peaks against the known amount of nucleic acid on the filter. The concentration of the probe must be in excess over that on the filter and the autoradiograph should not be overexposed. An example of densitometric quantitation of a blot is given in *Figure 5*. Note that the curve relating intensity of signal (area under the peak) to the amount of RNA in the dot is only linear for a restricted range of amounts of filter-bound RNA. So, for the probe used in *Figure 5*, quantitation can be carried out only over the range 0 − 6 μg RNA per dot since beyond this the filter-bound sequences are in excess of this probe. The curve in *Figure 5c* does not reach a plateau in the range analysed because the size of the dots is not uniform.

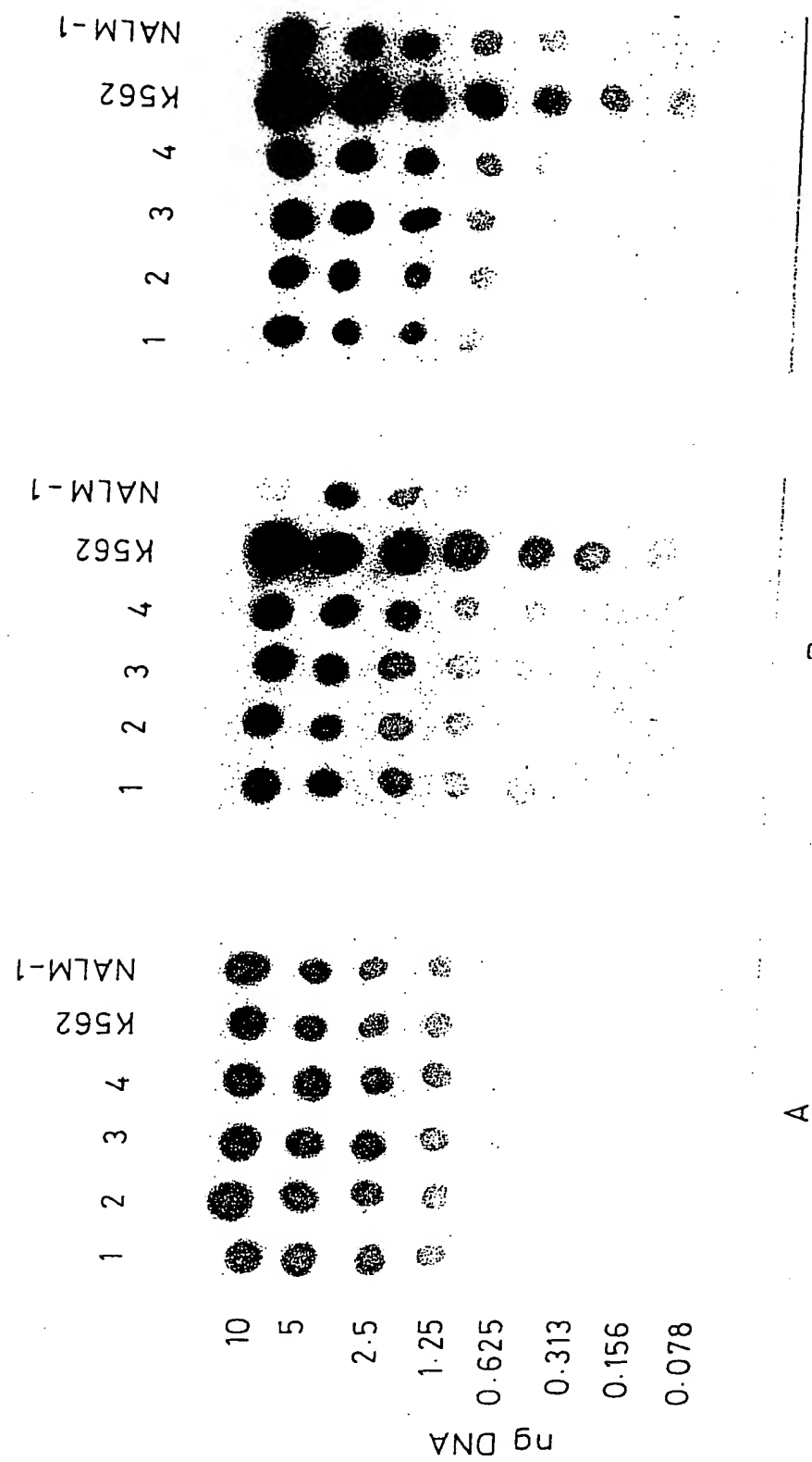

Figure 4. Genomic DNA dot blots. Replicate nitrocellulose filters containing the indicated amounts of genomic DNA from a dilution series were probed with (A) ^{32}P-labelled c-*sis* oncogene, (B) c-*abl* oncogene and (C) Ig V_λ DNA. Lanes 1–4 contained DNA from peripheral blood of chronic myeloid leukaemia (CML) patients, 5 and 6 contained DNA from CML cell lines K562 and NALM-1, respectively.

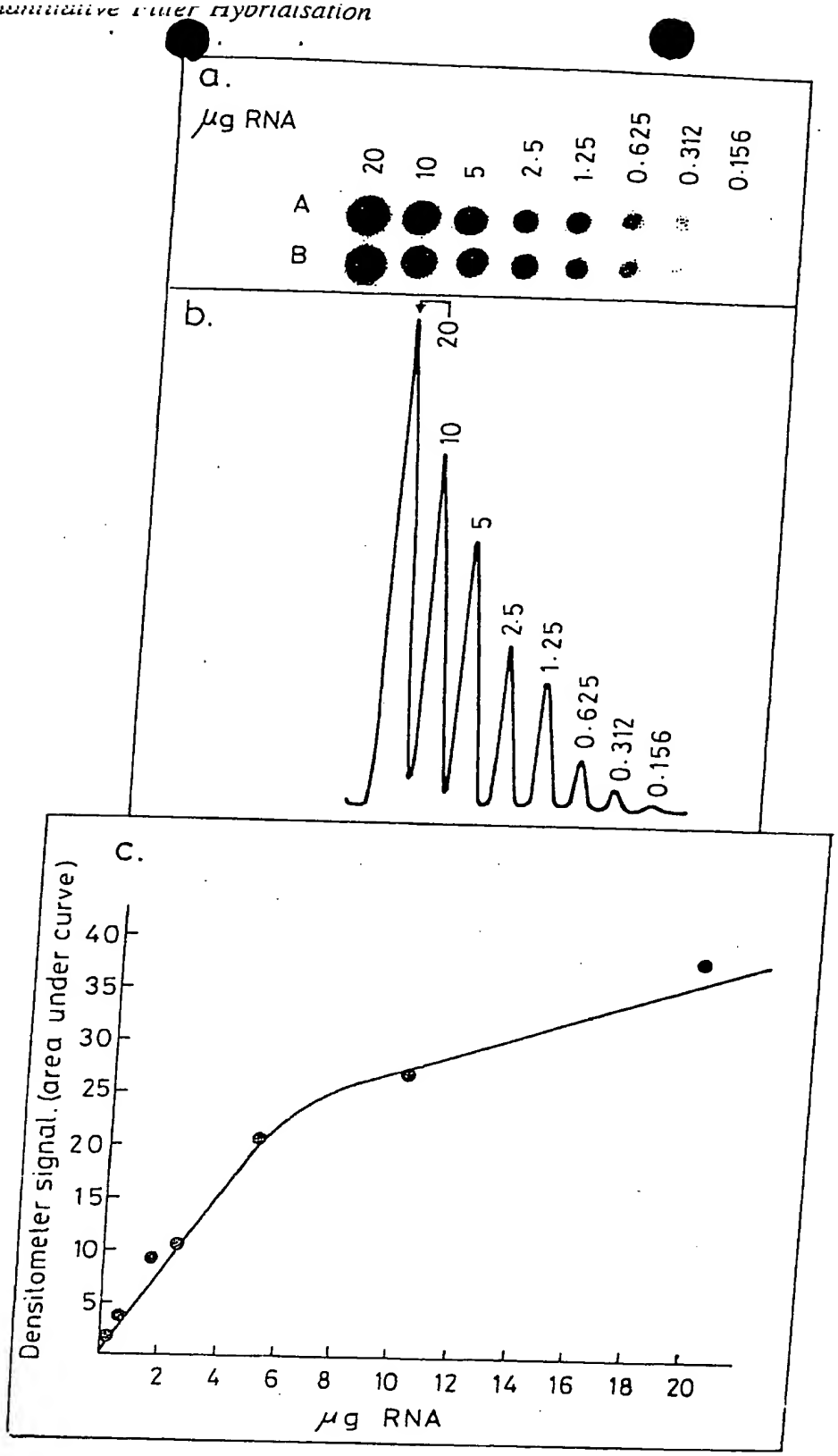

Figure 5. Quantitation of RNA dot blots. (a) Serial dilutions of RNA from patient 2 in *Figure 3* were prepared and applied in duplicate (A,B) to a nitrocellulose filter. The filter was hybridised with the Ha-*ras*-1 probe (*Figure 3*). (b) Densitometric scan made across lane A. (c) The relationship between the amount of RNA in each dot and the area under the densitometric peak for each dot.

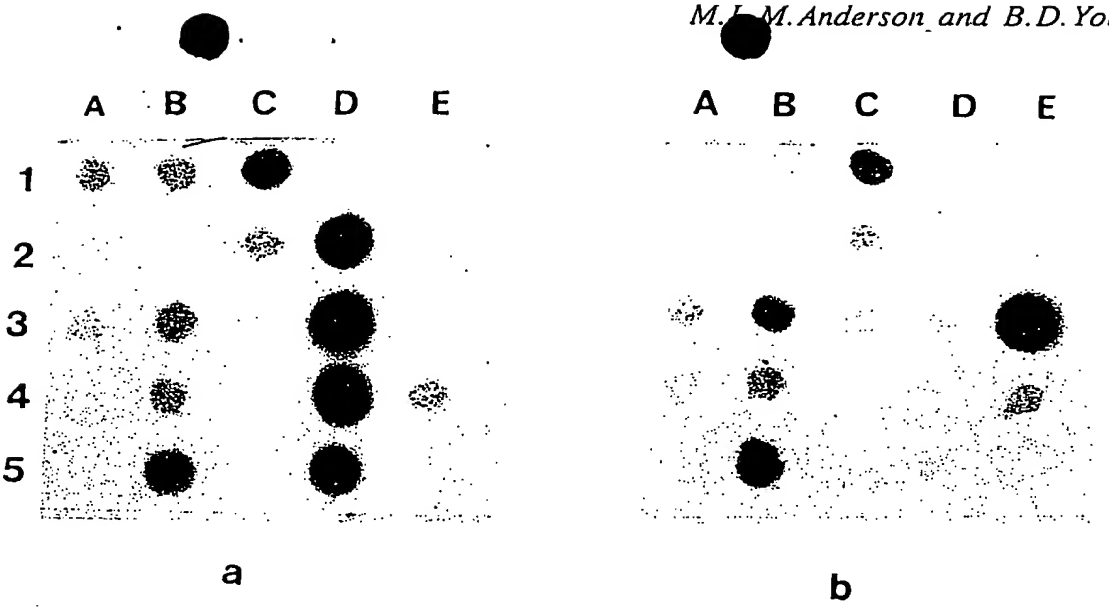

Figure 6. Plasmid DNA dot blots. Duplicate filters contained cloned recombinant plasmid cDNAs derived from mRNA of a patient suffering from acute non-lymphocytic leukaemia (ANLL). Probes were [^{31}P]cDNA derived from unfractionated mRNA of (a) ANLL and (b) chronic lymphocytic leukaemia (CLL) patients. The DNA bound to the filter was in significant excess over the probe. Data from M. Warnock, with permission.

If the hybrids are sufficiently radioactive, the dots can be cut out and counted in a liquid scintillation counter. This means, of course, that the filter cannot be re-hybridised.

10.3 Quantitative Analysis of Nucleic Acid Complexity

As we have seen, the rate of hybridisation is inversely proportional to the complexity of nucleic acid for both solution hybridisation and nucleation-limited filter hybridisation although not for diffusion-limited filter hybridisation (Section 2). Reassociation kinetics in solution have been used extensively to analyse the complexity of DNA and RNA populations and it might be supposed that nucleation-limited filter hybridisation could be used for a similar purpose. However, filter hybridisation is not suitable for quantitative studies of complexity. This is because the rate of filter hybridisation is so low that it is difficult to obtain C_0t values high enough for single copy sequences to hybridise (see Section 2).

10.4 Measurement of Relative Abundance of RNA Transcripts

For high and medium-abundance classes, dot blot hybridisation can be used to measure the relative prevalences of different mRNA species (44,45). Cloned recombinant cDNAs are applied in dots to filters and hybridised with either labelled mRNA or the cDNA derived from it. Filter-bound DNA is in excess so the extent of hybridisation is a measure of the concentration of the cloned cDNA sequence in the mRNA probe. [The extent of reaction is a reproducible characteristic of each clone and not a function of the cloned insert length, at least between the limits of 400 – 1500 nucleotides tested (44,45)]. It is estimated that a clone must be represented to a level of at least 0.1% of the mass of mRNA to be detected (44,46). This is probably true for optimal reaction conditions, but in practice the lower limit is more likely to be nearer 0.5%. An example is shown in *Figure 6*. Recombinant cDNA clones were constructed using mRNA from a patient

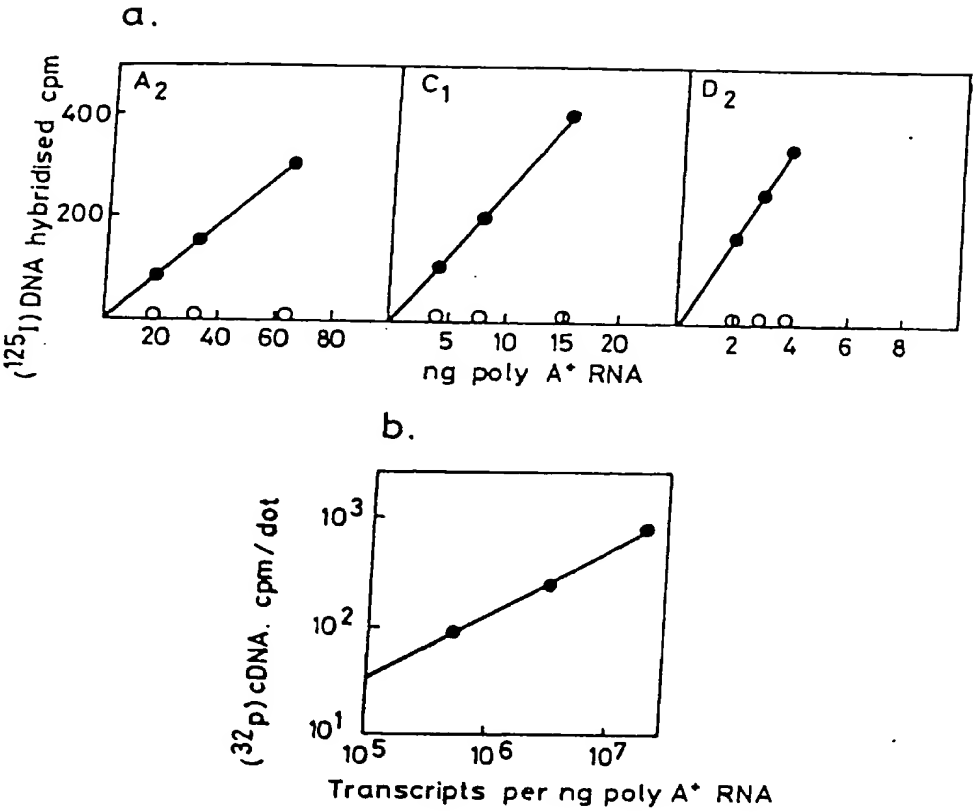

Figure 7. Prevalence analysis of mRNA transcripts. (a) Titration of cDNA clones with poly (A)$^+$ mRNA. Complementary strands (open and closed circles) of [^{125}I] cDNA recombinant plasmids A2, C1, and D2 (*Figure 6*) are hybridised separately in solution with the indicated amounts of poly(A)$^+$ RNA from ANLL cells. Reactions are carried out to 20 x $C_0t_{½}$ calculated with respect to the labelled DNA which is in sequence excess. The RNA-DNA hybrids formed in the reaction are analysed by resistance to nuclease S1. The slope of the lines was used to determine the number of transcripts per ng RNA. (Section 10.2).(b) Relationship between [^{32}P]cDNA dot blot hybridisation (*Figure 6*) and the number of transcripts per ng RNA for clones A2, C1 and D2.

suffering from acute non-lymphocytic leukaemia (ANLL). These were screened with [^{32}P]cDNA synthesised using unfractionated mRNA from an ANLL patient as template and with [^{32}P]cDNA complementary to unfractionated mRNA of a patient suffering from chronic lymphocytic leukaemia (CLL). Recombinant clones representing mRNAs common to the two diseases (e.g., B5, C1), and their relative abundance, can be easily identified and distinguished from those apparently specific to ANLL (e.g., D2, D3. D4, D5).

By using a calibration curve, the extent of hybridisation of [^{32}P]cDNA to each filter-bound recombinant can be used to determine the actual number of transcripts of mRNA (47,48). From the dot blot results (*Figure 6*), at least three cDNA clones whose representation in mRNA differs in abundance over a wide range are selected (e.g., clones A2. C1 and D2). Separated single strands of these recombinant DNAs are radiolabelled and hybridised separately in solution to different amounts of mRNA. The concentration of reactants is adjusted such that recombinant DNAs are in sequence excess. The reactions are carried out to about 20 x $C_0t_{½}$. The radioactivity in nuclease S1-resistant hybrids is determined and plotted against the amount of mRNA added to the reaction.

As expected, only one of the separated strands of DNA hybridises with the mRNA (*Figure 7a*). The number of transcripts per ng mRNA is then determined from the slope of the line using the relationship:

$$T = \frac{fN}{LS \times 350 \times 10^9}$$

where T is the number of transcripts per ng mRNA, f is the slope of the titration curve (c.p.m./ng mRNA), N is Avogadro's Number (number of molecules/ mol), L is the length of hybrid (i.e., cDNA insert length in nucleotides), S is the specific activity of the labelled cDNA (c.p.m./ng), and 350 is the average molecular weight of a ribonucleotide.

The appropriate values for clones A2, C1 and D2 of *Figure 7a* are:

Clone	*Specific activity* (S)	*Length of insert* (L)	*Slope* (f)	*No. of transcripts/ng mRNA* (T)
A2	2.0×10^4	800	4.7	5×10^5
C1	1.5×10^4	1000	26.3	3×10^6
D2	1.0×10^4	750	89.4	2×10^7

Using these values of T, a graph is plotted of the number of transcripts against the radioactivity in the hybrids on the dot blots. A linear relationship is obtained (*Figure 7b*). From this graph, the number of transcripts of any other recombinant on the dot blot matrix can easily be obtained.

11. DETERMINATION OF OPTIMAL REACTION CONDITIONS

11.1 Buffer Composition and Temperature

To determine the optimal reaction conditions, prepare replicate dot blots. Hybridise some under different hybridisation conditions, keeping the washing conditions constant, and monitor the effects. Then hybridise other dot blots under optimal hybridisation conditions and vary the washing conditions. For an extensive analysis of the effects of altering conditions on the hybrids formed, the reader is referred to references 3 – 5 and 43. The following is a rough guide.

(i) Reaction conditions which favour the detection of well-matched hybrids involve high temperatures of hybridisation (65 – 68 °C in aqueous solution and 42 °C in 50% formamide) combined with washing at high temperatures (5 – 25 °C below T_m) and at low salt concentrations (0.1 x SSC).

(ii) To detect poorly-matched hybrids, filters should be hybridised in solutions containing formamide (20 – 50%) at 35 – 42 °C but washed at high salt concentrations at an intermediate temperature (e.g., 2 – 6 x SSC at 40 – 60 °C). Again, conditions may have to be determined empirically. It should be remembered that both closely-related and distantly-related sequences will be detected under these conditions.

(iii) To distinguish between closely- and distantly-related members of the same family, conditions must be found which are permissive for some sequences and stringent for others. As we have already seen (Section 5), distant homologies are best detected when the ratio of rate constants for hybridisation of cross-hybridising to self-hybridising species is high, whereas closely-related species are most easily detected when the ratio is low. In practical terms, this means that for distantly-related hybrids low temperatures of incubation are used, whereas for closely-related hybrids high temperatures are best. The time of incubation is very important since the effective discrimination between closely- and distantly-related hybrids is highest with very short times of incubation and deteriorates very rapidly thereafter (Section 5). If short incubations do not give a sufficiently high hybridisation signal, then longer times can be used with excess filter-bound nucleic acid.

11.2 Time Period of Incubation

It is difficult to give a precise time period for hybridising filters. Filter hybridisations tend not to go to completion. As described above, the rate of hybridisation on filters is about 10 times slower than that for solution hybridisation of the same DNAs (9), so it is difficult experimentally to reach the very high C_0t values required for complete hybridisation. Prolonged incubation does not necessarily increase the extent of hybridisation because:

(i) more and more probe reassociates
(ii) at the high temperatures involved, sequences leach off the filter if they are not covalently bound
(iii) the probe is gradually degraded.

Addition of formamide to the hybridisation solution allows lower temperatures to be used and thus incubation times can be extended, but there is still the problem of probe reassociation unless a single-stranded probe is used. Furthermore, steric constraints prevent all the bound sequences being nucleated effectively. Under optimal conditions, no more than 80% of a single sequence probe appears in hybrids (9) and so an even smaller proportion of more complex probes will be hybridised.

In practice, for double-stranded DNA probes, there is no need to proceed for longer than to allow the probe in solution to achieve $1-3 \times C_0t_{1/2}$. After incubation for $3 \times C_0t_{1/2}$, the amount of probe available for additional hybridisation to sequences on the filter is negligible. The following useful guideline is taken from ref. 46. In 10 ml hybridisation solution, 1 μg denatured double-stranded probe with a complexity of 5 kb will reach $C_0t_{1/2}$ in 2 h. To determine the number of hours (n) needed to achieve $C_0t_{1/2}$ for renaturation of any other probe, the appropriate values can be substituted in the following equation:

$$n = \frac{1}{X} \times \frac{Y}{5} \times \frac{Z}{10} \times 2$$

where X is the weight of the probe added (in μg), Y is its complexity (which for most probes is proportional to the length of the probe in kb) and Z is the volume of the

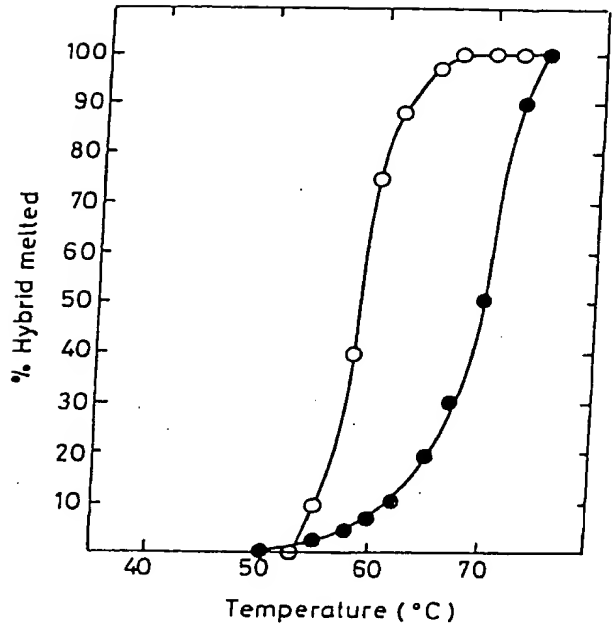

Figure 8. Melting profile of dot hybrids. Replicate filters containing dots of linearised DNA are hybridised to homologous ^{32}P-labelled DNA (○) or RNA (●). Hybridisation, in a formamide-containing solution, is carried out to 20 x $C_0t_{1/2}$. The DNA bound to the filter is present in significant excess. After hybridisation each filter is washed in the same formamide-containing buffer as used for hybridisation, with stepwise increases in temperature. The filters are counted by Cerenkov counting between each washing step to determine the percentage of hybridised [^{32}P]DNA or [^{32}P]RNA which has eluted (melted) at each temperature.

reaction (in ml).

For quantitative dot blots (e.g., *Figure 6*) the time period of incubation, the concentration of probe and the amount of nucleic acid bound to the filter should all be adjusted such that there is low fractional hybridisation of both the probe and filter-bound sequences. This is to ensure that the proportion of the probe hybridising increases linearly with time.

Factors affecting the selection of a time of incubation suitable for discrimination between related sequences are discussed in Section 5.2 [see also Section 11.1 (iii)].

12. MEASUREMENT OF T_m

The DNA or RNA sample is applied in a dot to a filter and hybridised with a labelled DNA or RNA probe as appropriate. It is then washed at the same temperature in a solution of the same composition as was used for the hybridisation. This removes unhybridised and non-specifically-bound probe. For melting of the hybrid, the filter is incubated for 10 – 15 min in a small volume of the same buffer at progressively higher temperatures. The buffers used must be pre-heated to the required temperature. Between melting steps, the filter is counted by Cerenkov counting, making sure that it does not dry out at any stage in order to prevent irreversible binding of the probe. Replicate filters can be hybridised at different criteria or washed in solutions of different ionic strength. *Figure 8* shows the melting temperature profiles to be expected from homologous DNA-DNA and RNA-DNA hybridisations. Note that in formamide-containing

solutions RNA-DNA hybrids are more stable than the corresponding DNA-DNA hybrids and so dissociate (melt) at higher temperatures. The exact temperature difference will depend on the base composition of the nucleic acids involved, the size of the probe and the degree of relatedness of the two hybridising species. In the example given in *Figure 8*, the difference in T_m is 11°C.

For a single nucleic acid species hybridising to itself, the hybrid melts over a very narrow temperature range and the T_m is the same irrespective of the incubation temperature, T_i. However, when the same nucleic acid is probed with a complex mixture of sequences which have varying degrees of relatedness, the T_m profile depends on the reaction conditions. For hybrids formed at low criterion (T_m −25°C), the melting profile is broad because both well-matched and poorly-matched hybrids are formed. They melt at different temperatures, so the overall melting profile, which is a composite of the contributions of all the hybrids, will reflect this. At high criterion (T_m −8°C), only hybrids with a high degree of homology form so they melt over a very narrow temperature range. The melting profile is also broad when variable length probes are used. This is most apparent at short average lengths of hybridised probe in accordance with the empirical relationship:

$$T_n - T_m = \frac{650}{L}$$

where L is the length of the probe in nucleotides, T_m is the melting temperature of the short hybrid and T_n is the melting temperature of long DNA molecules (17).

The procedure of stepwise melting of hybrids described above for T_m measurement can be extended to investigate the degree of relatedness of different sequences and can be applied to many samples at once. An array of dots on a single filter are hybridised to a labelled probe and the extent of similarity to the probe is evaluated by stepwise melting and autoradiography (3). The more mismatched a hybrid is, the lower the temperature at which it will melt. Thus differences in the intensity of signal of the dots can be interpreted in terms of the degree of relatedness of the different sequences.

13. RE-USE OF FILTERS AND PROBES

13.1 Filters

In many cases, re-probing the same filters with a series of different probes yields valuable information. Filters can be re-probed several times — the exact number being dependent on the type of filter and the incubation conditions to which the filter has already been exposed. Nitrocellulose filters which have been exposed to high temperatures for hybridisation and washing can be used about two to four times before falling apart. Filters exposed to the less harsh conditions of hybridisation at lower temprature in the presence of formamide can be used many more times. Nylon filters can be used indefinitely without disintegration and so, because of their superior durability, they are preferred to nitrocellulose for multiple probings.

Before re-probing a filter with a new probe, it is first necessary to strip off the old probe and to monitor that the treatment has been effective. This can be achieved for both DNA and RNA dot blots as follows:

(i) Transfer the damp filter to a plastic box containing 200 ml of 5 mM Tris-HCl. pH 8.0, 0.1 mM EDTA, 0.05% sodium pyrophosphate, 0.1% Denhardt's solution (see *Table 3*, footnote c for composition) at 65°C. Incubate for 1 h with gentle agitation.

(ii) Discard the wash buffer and repeat step (i).

(iii) Check by autoradiography as described in Section 10.1 that the probe has been removed.

An alternative procedure for DNA dot blots only is given below.

(i) Wash the filter twice, for 10 min each wash, in 50 mM NaOH at room temperature.

(ii) Wash and neutralise the filter by incubating it (5 min each time) in five changes of TE buffer, pH 7.5 (see *Table 1*, footnote a) at room temperature.

This procedure cannot be carried out successfully for RNA dot blots since the NaOH hydrolyses the filter-bound RNA.

Unfortunately there are several potential problems with re-using filters:

(i) Loss of sensitivity. Prolonged use of both nylon and nitrocellulose filters leads to gradual reduction in sensitivity through loss of filter-bound nucleic acid (Section 6.1). This is not a problem if the nucleic acid has been covalently bound to the filter matrix.

(ii) Irreversible binding of the previous probe if the filter was allowed to dry.

(iii) Incomplete removal of the previous probe even if the filter was kept wet throughout its use. This can give misleading results if single-stranded tails of probe remaining on the filter are complementary to sequences of the new probe. For example, if the first probe is a recombinant DNA (vector and insert) and the second is also a recombinant DNA with a different insert, hybridisation may occur through plasmid sequences and this will obscure the hybridisation of the second probe insert sequences. Therefore, it is recommended that all inserts are excised from vectors before labelling as probes.

13.2 Probes

Normally only a small fraction of the probe is used up during hybridisation, so probes can be re-used until they are degraded or have decayed to too low a specific activity. To re-use the probe (now in the hybridisation solution) it must be denatured again by heating to a temperature above its T_m. For aqueous solutions, this can be done by incubating in a boiling water bath for 10 min. For formamide-containing solutions, heat at 70°C for 30 min. The newly-denatured probe can now be added to a second filter which has been pre-hybridised under standard conditions.

14. PROBLEMS

Most hybridisation experiments using filter-bound nucleic acids employ radioactive probes and so problems of only this type of investigation are covered here.

(i) The autoradiograph of the filter is black all over:

(a) At some stage during hybridisation or washing, the filter was allowed to

dry. It will probably be necessary to strip the filter (Section 13.1) and re-hybridise.

(b) The probe is 'dirty'. It may be contaminated by traces of agarose. Either re-purify the nucleic acid from which the labelled probe was derived and prepare a new probe, or pass the labelled probe through a nitrocellulose filter which has been pre-treated in 10 x Denhardt's solution or through a mini NACS column (BRL).

(c) An inappropriately low hybridisation and/or washing temperature was used.

(ii) The autoradiograph of the filter is black in parts:

(a) Part of the filter dried out; see above.

(b) The filter was handled with bare hands. Grease marks from fingers trap probe. Wear disposable plastic gloves in future.

(iii) The autoradiograph has black dots in random locations:

(a) The unincorporated precursors were not completely removed from the probe. See correct procedure in relevant section of Chapter 2.

(b) Air bubbles were not completely removed from the bag during hybridisation. (This may not matter if a shaking water bath is used, but the effect may be quite troublesome if the bag is not agitated.)

(c) Dust or dirt on the filter. Filter all solutions before use in future.

(iv) The signal is lower than expected:

(a) Was the correct binding procedure used? Nitrocellulose and nylon filters use different binding protocols (see Section 6.1).

(b) The probe was degraded. This is most likely to happen with RNA probes.

(c) The double-stranded probe was not denatured (see Section 8.2.2).

(d) The hybridisation and/or washing conditions were too stringent so that the hybrids either did not form at all, or were dissociated.

(e) The specific activity of the probe was too low.

(f) The hybridisation time was too short.

(g) The filter was not exposed to film for long enough.

(v) A 'negative' effect is obtained, that is, the background of the autoradiograph is black with clear dots. Too high a concentration of [^{32}P]probe was used.

(v) The filter fell apart. This is most likely to occur with nitrocellulose filters.

(a) During binding of DNA to the filter, the alkali was not properly neutralised thus making the filter yellowish in colour and very brittle.

(b) After repeated use, the filter becomes brittle despite correct procedures. Prepare new filters.

15. ACKNOWLEDGEMENTS

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Biochemistry

Base composition-independent hybridization in tetramethylammonium chloride: A method for oligonucleotide screening of highly complex gene libraries

(DNA melting)

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ABSTRACT **An oligonucleotide hybridization procedure has been developed that eliminates the preferential melting of A·T versus G·C base pairs, allowing the stringency of the hybridization to be controlled as a function of probe length only. This technique, which uses tetramethylammonium chloride, is especially helpful whenever a highly complex library is screened with a pool of oligonucleotide probes, which usually vary widely in base composition. The procedure can also be applied advantageously whenever an exact match to an oligonucleotide probe is desired, such as in screening for clones having as little as a single-base alteration generated by *in vitro* mutagenesis.**

Short oligonucleotide probes [range, 14–20 base pairs (bp)] are commonly used to screen libraries of cloned DNA for genes of interest (1–3). Typically, these probes are pools representing all possible codon choices for a short amino acid sequence. Although this method has been successful, there is considerable uncertainty in the hybridization conditions because the binding of the oligonucleotides depends on two factors: (*i*) the length of the hybrid formed and (*ii*) the G·C content of the probe. Empirically determined formulas allow for estimation of the oligonucleotide dissociation temperature (T_d) (4); however, these methods can be unsatisfactory when screening with pools of oligonucleotides. Although the length of the probes in the pool is constant, the individual probes differ considerably in G·C content, making suitably stringent and selective hybridization conditions difficult to find for all members of the pool. Thus, a large number of false positives can occur when screening highly complex libraries for genes of low abundance.

We describe here the use of tetramethylammonium chloride (Me_4NCl) in the hybridization of oligonucleotide probes to eliminate the dependence of T_d on the G·C content of the probe, reducing the problem to a simple dependence on length of the hybrid. Tetraalkylammonium salts were found some years ago to bind to A+T-rich polymers of DNA (5) and have been used to abolish the preferential melting of A·T versus G·C base pairs for fragments of DNA (6). Me_4NCl binds selectively to A·T base pairs, thus displacing the dissociation equilibrium and raising the melting temperature. At 3.0 M Me_4NCl, this displacement is sufficient to shift the melting temperature of A·T base pairs to that of G·C base pairs (6). For the melting of long DNA, this shift results in a remarkable sharpening of the melting profile. Natural DNAs that melt over a range of 5 to 10°C in the presence of Na^+ melt within 1°C in Me_4NCl (6–8). The data presented here show the utility of hybridizations with Me_4NCl when oligonucleotide probes are used. The T_d values of probes from 11 to >1000 bp have been determined so that hybridization conditions for probes of various lengths can be chosen easily. This method is applicable to a variety of circumstances in which an exact match to a probe is desired.

METHODS

DNA Synthesis, Binding, and Labeling. In this procedure, nitrocellulose filters are used routinely with DNA spotted directly or bound (9) from bacteriophage λ or M13 plaques or from plasmid-containing bacterial colonies. Filters removed from bacteriophage or bacterial colony plates are treated with 0.5 M NaOH/1 M NaCl, which is neutralized with 1 M Tris·HCl, pH 7.5/1.5 M NaCl. DNA spots are made by denaturing the sample with 0.3 M NaOH, neutralizing with 0.6 M Tris·HCl, pH 7.4/1.5 M NaCl, and immediately spotting on nitrocellulose filters previously soaked in 3.0 M NaCl/0.3 M Na citrate (20× NaCl/Cit) and dried. The DNA-containing filters are baked for 2 hr at 60–80°C in a vacuum oven. Oligonucleotide probe pools were synthesized from trimers by the triester method (10). Pools 9.3 and 9.4 were end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (9).

Hybridization and Me_4NCl Wash Procedure. In this procedure, an initial nonstringent hybridization with radiolabeled probe is followed by washing with 3.0 M Me_4NCl to control the stringency of the hybridization. The filters are prehybridized in 6× NaCl/Cit/50 mM sodium phosphate, pH 6.8/5× Denhardt's solution (1× Denhardt's solution is albumin/polyvinylpyrrolidone/Ficoll, each at 0.2 mg/ml) containing boiled sonicated salmon sperm DNA at 0.1 mg/ml for 4–16 hr at 37°C. The filters are hybridized overnight at 37°C in the same solution plus dextran sulfate at 100 mg/ml and the pool of end-labeled oligonucleotide probes (each at 180 pM; 560,000–1,680,000 cpm/pmol) (11). [For a 17-mer probe this is 1 μg/liter (100–300 cpm/pg). For a pool of 16 probes, the probe concentration is 16 μg/liter.] The filters are rinsed three times with 6× NaCl/Cit at 4°C and washed twice for 30 min with 6× NaCl/Cit at 4°C. The filters are then rinsed with the Me_4NCl wash solution at 37°C to remove the NaCl/Cit [the NaCl/Cit must be substantially removed because Na^+ will compete for Me_4N^+ binding (12)] and washed twice for 20 min with the Me_4NCl wash solution at the desired temperature. In screening experiments, we typically use a temperature 2–4°C below the T_d shown in Fig. 3. The wash temperature needs to be well controlled (±1°C); suitable heat exchange can be obtained only in a shaking or circulating water bath. The Me_4NCl wash solution is 3.0 M

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Abbreviations: bp, base pair(s); T_d, dissociation temperature; Me_4N^+, tetramethylammonium ion; Et_4N^+, tetraethylammonium ion; NaCl/Cit, 0.15 M NaCl/0.015 M Na citrate.

Me_4NCl/50 mM Tris·HCl, pH 8.0/2 mM EDTA containing NaDodSO$_4$ at 1 mg/ml. Me_4NCl is purchased from Aldrich and made up as a nominal 5 M stock solution. Since Me_4NCl is hydroscopic, the actual molar concentration (c) is determined from the refractive index (η) by the formula $c = (\eta - 1.331)/0.018$ (12). The concentration needs to be fairly precise since the T_d varies with the Me_4NCl concentration (6).

The prehybridization and hybridization described above are used for oligomers in the range 14–50 nucleotides. For shorter oligonucleotides, a room temperature hybridization is preferable. For oligonucleotides of ≥ 50 nucleotides, the prehybridization and hybridization are as above except that formamide at 400 ml/liter is included at 37°C. In the range 46–75 bp, we have compared hybridization with and without formamide; the two give equal results. Nitrocellulose filters become fragile in washes at 85°C and above. If frequent use is made of high-temperature washes, it is possible to use 2.4 M tetraethylammonium chloride (Et_4NCl) rather than 3.0 M Me_4NCl. Et_4NCl has the same desirable effect on DNA melting as Me_4NCl [at least with long DNA (6, 7)], but the entire melting profile is shifted 33°C lower. Although with Et_4NCl the melting temperature of short oligonucleotides is inconvenient (below room temperature), it could be useful for 50- to 200-bp probes.

RESULTS AND DISCUSSION

The results of hybridization and melting of four oligonucleotide probes in the commonly used 6× NaCl/Cit system (1, 2, 11, 13) are compared with those in 3.0 M Me_4NCl in Fig. 1. These probes are 17-mers, and their hybridization is determined by the stretch of contiguous match, the hybridizing length, rather than by the percentage homology because even a single internal mismatch in probes of this length lowers the T_d by 5–10°C (refs. 11 and 13; unpublished observations). A series of DNA spots was hybridized under nonstringent conditions and washed at temperature intervals of 2 or 3°C. As shown in Fig. 1*A*, the melting in 6× NaCl/Cit does not depend linearly on the hybridizing length. In particular, the probe with the 13-bp hybridizing region melts some 5°C higher than the 15-bp probe and at about the same temperature as the 16-bp probe. This is because the probe with the 13-bp hybridizing region is 69% G·C while the 15- and 16-bp probes are 40 and 44% G·C. These melting results in 6× NaCl/Cit contrast with those in 3.0 M Me_4NCl (Fig. 1*B*) in which the probes melt according to their hybridizing length. These results illustrate that the preferential melting of A·T base pairs is abolished in oligonucleotide hybridizations in 3.0 M Me_4NCl, as previously shown for long DNA (6). The T_d values derived from the data in Fig. 1 are plotted as a function of hybridizing length in Fig. 2.

To extend the utility of the method over a wide range of probe lengths, the T_d values in 3.0 M Me_4NCl were determined as a function of length for 18- to 1374-bp restriction fragments of pBR322. These fragments were end-labeled, hybridized to a series of pBR322 DNA dots, and washed at various temperatures with 3.0 M Me_4NCl. The T_d, length, and G·C content of these fragments are shown in Table 1. When plotted as a function of length, the T_d values lie on a smooth curve from 45 to 93°C in spite of the widely varying G·C content (31–66%) of the probes (Fig. 3). This further demonstrates the lack of dependence of the T_d on the G·C content. The T_d is essentially independent of length above 200 bp, and the limiting T_d of 93°C agrees with that reported for long DNA (6, 7). From either Fig. 3 or Table 1 one can determine the hybridization wash temperature so that the stringency can be based solely on the probe length.

The utility of hybridization in Me_4NCl is perhaps best illustrated by the following example based on our own experience: A pool of sixteen 17-mers representing all possible codons for the protein sequence Glu-Cys-Trp-Cys-Gln-

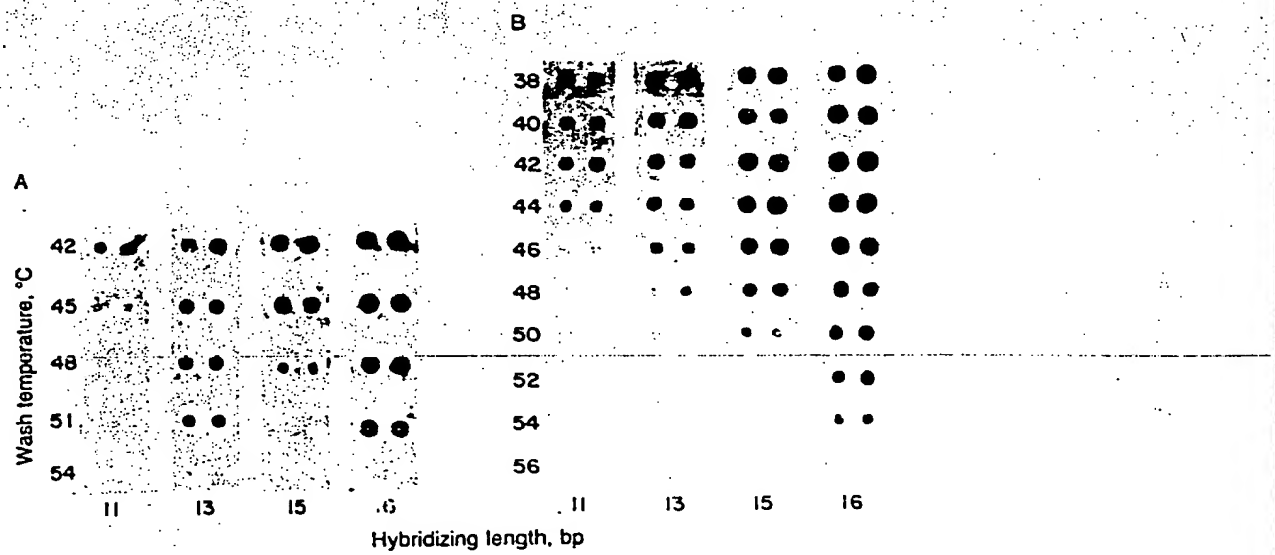

FIG. 1. Hybridization of oligonucleotides of various hybridizing lengths washed in 6× NaCl/Cit or 3.0 M Me_4NCl at increasing temperatures. Spots containing 125–250 ng of bacteriophage λ20a or λ21a DNA were made on nitrocellulose membranes, and these DNA spots were hybridized to ^{32}P-labeled probe pools 9.3 or 9.4. Pool 9.3 contains sixteen 17-mer probes of the sequence 5′ G-C-$\frac{C}{T}$-T-G-$\frac{A}{G}$-C-A-C-C-A-$\frac{A}{G}$-C-A-$\frac{C}{T}$-T-C. Pool 9.4 contains sixteen 17-mers with the sequence 5′ T-A-$\frac{C}{T}$-T-G-$\frac{C}{T}$-T-T-C-C-A-$\frac{A}{G}$-A-A-$\frac{C}{T}$-T-C. λ20a and λ21a are clones isolated from a bovine genomic library (14) by conventional hybridization with 6× NaCl/Cit washes to pools 9.3 and 9.4 (unpublished data). Pool 9.3 has a contiguous match of 11 bp to λ21a, A-*C-T-T-G-A-C-A-C-C-A-A*-A-C-*T*-C-A (the nucleotides in italic type are those of the λ21a sequence that match the probe), and a contiguous match of 13 bp to λ20a, *G-C-T-T-G-G-C-A-C-C-A-G-C-T-T*-G-C. Pool 9.4 has a 15-bp match to λ21a, *T-G-T-T-G-C-T-T-C-C-A-G-A-A-T-T-C*, and a 16-bp match to λ20a, A-*A-C-T-G-C-T-T-C-C-A-A-A-A-C-T-C*. Duplicate hybridized spots were washed at the indicated temperatures in 6× NaCl/Cit containing NaDodSO$_4$ at 1 g/liter twice for 10 min (*A*) or in 3.0 M Me_4NCl (*B*) as described in *Methods*. Spots are shown after autoradiography overnight at −70°C with DuPont Lightning Plus intensifying screens.

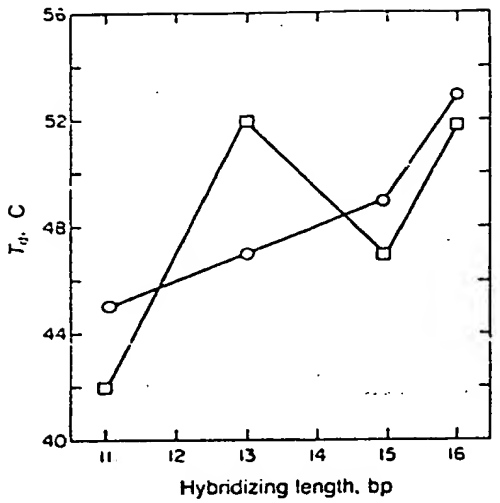

FIG. 2. T_d of oligonucleotide probes as a function of hybridizing length in 6× NaCl/Cit (□) or 3.0 M Me_4NCl (○). T_d values were determined visually from the data in Fig. 1 as the temperature at which half the intensity remained and plotted as a function of hybridizing length. T_d values are used rather than melting temperature (T_m) values, which are determined under conditions of thermodynamic equilibrium.

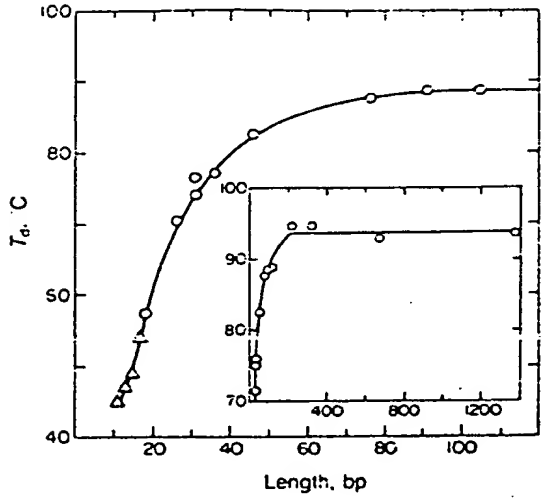

FIG. 3. T_d in 3.0 M Me_4NCl as a function of probe length. A series of pBR322 DNA spots was made on nitrocellulose membranes, and these were hybridized and washed with 3.0 M Me_4NCl. Washes of the duplicate spots were carried out at 3°C intervals. Hybridization probes of various lengths were made by cutting pBR322 DNA with *Sau*3AI, treating with alkaline phosphase, labeling with T4 polynucleotide kinase and [γ-^{32}P]ATP, and isolating the labeled fragments from a polyacrylamide gel (9). These double-strand probes were boiled before hybridization. From an autoradiogram of the hybridized spots (analogous to that shown in Fig. 1), a T_d was determined and plotted for each fragment (○). Also shown are melting data in Me_4NCl from Fig. 2 (△).

Ala was synthesized (probe 9.3; see Fig. 1). This pool consists of oligonucleotides ranging in G-C content from 47 to 71%. In 6× NaCl/Cit, a temperature that allows hybridization of the probe having the lowest G-C content will also allow hybridization of regions as short as 12 or 13 bp in the probes having the highest G-C contents. When screening a highly complex library, such as those derived from mammalian genomes, this can lead to a large number of false positives. The expected number of hybridizing sequences per haploid genome at random, N, is approximately

$$N = C(2)(n - h + 1)(p)/4^h,$$

where C = complexity of the genome in bp, n = probe length, h = contiguous hybridization match, and p = pool size (see *Appendix* for derivation). Thus, for a pool of sixteen 17-mers where matches as short as 13 are allowed ($C = 3.3 \times 10^9$, $n = 17$, $h = 13$, $p = 16$), the expected number of clones at random is 8000. These clones represent a large number of false positives. However, with Me_4NCl, a hybridization temperature can be selected in which only 17- and perhaps some 16-bp matches are allowed (52–54°C). In this case, the expected number of random clones is only 6 (or 49 for 16-bp matches), a number small enough to be searched through for the correct clone by DNA sequence analysis or by a precise melting in Me_4NCl as described below. As noted in the *Appendix*, the application of the formula for N to hybridization in 6× NaCl/Cit is not quite correct because all matches of length 13 (not just those of high G-C content) are counted. Thus, perhaps only 1000–4000 positives would be found with 6× NaCl/Cit hybridization. In any event, the number of false positives due to probes of high G-C content can be reduced significantly by using Me_4NCl.

Table 1. Probe G-C content and T_d in 3.0 M Me_4NCl

Hybridizing length, bp	G-C content, %	T_d in 3.0 Me_4NCl, °C
11	45	44–45
13	69	47
15	40	49–50
16	44	53–54
18	44	57–58
27	63	70–71
31	58	74 (76)
36	53	77
46	54	82–83
75/78	48/42	87–88
91	66	88–89
105	31	88–89
207	55	94–95
317	65	94–95
665	46	93
1374	55	93–94

Probes having hybridizing lengths of 11–16 bp are described in the legend to Fig. 1. Those 18–1374 bp long are *Sau*3AI fragments of pBR322.

In addition to screening highly complex libraries, the Me_4NCl procedure may be adapted advantageously in a variety of other situations. For example, it can be used to select the best candidates for DNA sequencing. If a number of candidate clones have been obtained, those that match the probe most closely can be found by plating out replicas of the clone or spotting the DNA, hybridizing to an oligonucleotide probe, and washing with 3.0 M Me_4NCl at increasing temperature in the manner of Fig. 1. We have used six duplicate spots of the DNA and washed at 2°C intervals to find the clones that match the probe exactly, thus eliminating unnecessary DNA sequencing. In this way, the match with the probe can be determined to ±1 bp in the 14- to 20-nucleotide range. This technique can be especially precise if standards of known hybridizing length are included at the same time. Another application of this method has been in screening of M13 clones that had been mutagenized *in vitro* (15). In this procedure, a 20-mer having one or more centrally located base changes is synthesized and used to generate M13 plaques that are screened for an exact match

with the same oligonucleotide as the probe. Since even a single-base-pair internal mismatch decreases the T_d of the hybrid 5 to 10°C (11, 13), the correct mutant can be found by screening in Me_4NCl, using the wash conditions shown in Fig. 3. Furthermore, for different oligonucleotides, empirical determination of a suitable wash temperature is unnecessary. Finally, the method can be used to select one particular gene out of a closely related gene family. For example, a clone for the γ subunit of mouse nerve growth factor was selected from a family of closely related kallikrein-like cDNA clones by hybridization in Me_4NCl (16). In this case, the probe was a pool of thirty-two 14-mers based on protein sequence data.

In summary, hybridization in Me_4NCl is useful whenever an exact match with an oligonucleotide probe is desired. This method is being used routinely for oligonucleotide hybridization to bacteriophage λ or M13 plaques, plasmid-containing bacterial colonies, and DNA spots. While we have not yet used the procedure for genomic blot hybridization with oligonucleotide probes, it should be useful in detecting single-base mismatches in hybridizations to genomic DNA (17). Here again, the hybridization conditions would not need to be determined empirically.

APPENDIX

Derivation of N, the Approximate Number of Sequence Matches Expected per Haploid Genome at Random. Let C = complexity of the genome in bp, n = probe length, h = contiguous hybridizing length, and p = pool size; then, considering the genome as random sequence, $N = C(2)f$, where f is the frequency of some sequence at random. The frequency times the number of bp, C, times 2 (the sequence of interest could be on either strand) is N, the number of sequence matches. The frequency of a particular sequence of length n, where every nucleotide matches, is $1/4^n$. If only h contiguous bp of a probe of length n match, then the frequency of sequence match is $1/4^h$ times the number of contiguous length h matches in the probe. For $n = 17$ and $h = 15$, the number of contiguous matches is $(n - h + 1) = 3$. Thus, for a single probe of length n, where contiguous matches of length h are allowed, the frequency of a sequence match is $(n - h + 1)/4^h$. If a pool of p such probes is used, then $f = p(n - h + 1)/4^h$ and $N = C(2)(n - h + 1)p/4^h$.

The expected number of clones at random, N, given by this formula is about the number we have found in screening a bovine genomic library by using probe pools 9.3 and 9.4 (Fig. 1). We screened 750,000 plaques of a library representing 3.5 haploid genomes with 3.0 M Me_4NCl washes at 50°C and found 80 and 130 positives per haploid genome for the two pools. For these conditions, we would expect 16- and 17-bp matches with perhaps some matches as short as 15 bp. For $C = 3.3 \times 10^9$, $n = 17$, $p = 16$, and $h = 16$ or 15, the formula gives $N = 49$–295 expected random positives, approximately the number found. Application of this formula, based on the contiguous match, is especially appropriate for hybridizations in Me_4NCl where the match length can be controlled. Use of the formula with hybridization in 6× NaCl/Cit is less exact because the number of hybridizing sequences in a pool is affected by the G·C content.

This formula can also be used to estimate the number of random matches to be found in cDNA or other libraries. For this purpose, C can be set to the total number of bp screened (assuming the clones are independent), and the number of hybridizing clones expected at random can be calculated. Clearly, this will overestimate the number of randomly hybridizing clones for most cDNA libraries because some clones are represented many times. However, it can still be used to obtain a rough approximation of what to expect for a particular sized library, oligonucleotide probe, and hybridization stringency.

Note Added in Proof. Recently we have successfully used the Me_4NCl procedure for genomic blot hybridization. The frequency in the population of a single base polymorphism in the factor VIII gene (18) was determined by the hybridization of duplicate lanes of human DNA to two 21-mer probes that differ only at a single central residue. Wash conditions based on Fig. 3 (58°C) revealed a single band with no lane-specific background and clearly showed that 7 of 11 individual DNAs have one sequence while the remaining 4 have the other.

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